# DISSECTION, LOCALIZATION AND INTERACTION OF THE VP1, ABA, LIGHT AND NEGATIVE REGULATORY CIS-ELEMENTS IN THE *C1* GENE OF MAIZE

BY

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Abstract of Dissertation Presented to the Graduate School
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# DISSECTION, LOCALIZATION AND INTERACTIONS OF THE VP1, ABA, LIGHT AND NEGATIVE REGULATORY CIS-ELEMENTS IN THE *C1* GENE OF MAIZE

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The *C1* regulatory gene of the anthocyanin pathway of maize is regulated by a combination of developmental and environmental signals. The *Viviparious* (*Vp1*) gene is required for expression of the *C1* in the developing seed of maize. Using protoplast electroporation and particle bombardment based expression assays, we have identified cis-acting elements for abscisic acid (ABA), VP1, light and a potential negative regulatory element within the region 150 bp upstream of transcription start site in *C1* promoter. The cis-elements for VP1 and ABA regulation are overlapping and resolve into the Sph element (CGTCCATGCAT), critical for both VP1 and ABA activation, and an adjacent 5 bp (CGTGT) that is important only for ABA regulation. We also show that the *C1* promoter and the promoter of homologous *pl* gene in maize plant are activated by light in

transient assays. Multiple photoreceptors including phytochrome are involved in this light activation. The cis-element required for light regulation is located within a 56 bp region which contains structural features that include a G-box motif, dyad elements related to the Unit I of UV light element in the *chs* gene of parsley. This minimal light element is functional in both seed and plant tissues and shares high homology to upstream sequence of the *pl* gene. Deletion of a negative regulatory element at -44, which also involves a G-box-like sequence, generally derepresses both basal expression and activated expression. However, deletions in this region affect expression only in developing and germinating seed tissues, indicating that the negative effect is seed specific. Whereas VP1, ABA and negative regulatory factors affected *C1* regulation only in seed tissues, light activation of *C1* was detected in all tissues tested.

# INTRODUCTION

Anthocyanins are a subclass of the flavonoids which comprise a large group of secondary metabolites in higher plants. The flavonoids synthesized in various plant tissues fulfill varied physiological functions as UV protectants, pollinator attractant, signal molecules and antibiotics for plant defense (Harborne 1986). In maize virtually every organ in the plant can form anthocyanin. The production of this pigment in different tissues is dependent on a combination of genotypic, environmental and developmental controls.

Synthesis of anthocyanins requires a number of genes that have both structural and regulatory roles (Dooner and Robbins 1991). The structural genes (*C1*, *A1*, *A2*, *Bz1* and *Bz2*) encode the enzymes in the biosynthetic pathway of anthocyanin that are required in every tissue to synthesize the pigment. Expression of these structural genes is transcriptionally controlled by the products of regulatory genes. These regulatory genes can be grouped into two families, *C1/Pl* and *R/B*, based on the evidence that genes within each family encode functionally equivalent proteins. For any particular tissue to synthesize the pigment requires a functional allele of the *C1 Pl* family and a functional allele of the *R/B* family. For example, anthocyanin synthesis in the aleurone of the seed requires the interaction of the seed-specific *C1* gene from *C1/Pl* family and *R-S* gene from *R/B* family.

During seed development, anthocyanins accumulate in scutellum and aleurone layer. This expression of anthocyanin requires, in addition to *C1* and *R1*, another regulatory gene, *Vp1*. Unlike *C1* and *R1* which affect only the anthocyanin pathway, the anthocyaninless phenotype of *vp1* mutant is associated with a failure of seed maturation. This pleiotropic effect of *vp1* implies that two independent programs in seed development -- seed maturation and anthocyanin biosynthesis -- may be coupled together by the function of *Vp1* (McCarty 1995).

The regulatory genes *Vp1*, *C1* and *R1* have been cloned by transposon tagging and characterized at the molecular level. In each case the protein sequences include domains with characteristics typical for transcriptional activators (Ludwig et al.1989, McCarty et al. 1991, Paz-Ares et al. 1987). The C1 and R1 proteins probably interact together on the promoters of the downstream structural genes (*A1*, *A2*, *Bz1*, *Bz2* and *C2*) to turn on the anthocyanin synthesis pathway (Dooner and Robbins 1991). However, particle bombardment experiments suggest that *C1*, but not *R1*, expression limits anthocyanin synthesis in the *vp1* mutant which implies an interaction between *Vp1* and *C1* (Hattori et al. 1992).

A hierachical relationship between *Vp1* and *C1* is supported by three lines of evidence. First, *C1* mRNA is absent in *vp1* mutant seed (McCarty 1989). Second, the peak expression of *Vp1* mRNA precedes the appearance of *C1* mRNA (McCarty et al.1989, 1991). Finally, over-expression of VP1 in maize protoplasts can transcritionally activate a chimeric reporter gene driven by the *C1* promoter (Hattori et al. 1992). This work also showed that the hormone abscisic acid (ABA) can cause activation of the reporter gene in the absence of VP1.

In addition to the requirement for a particular genotypic combination, environmental factors can qualitatively and quantitatively influence anthocyanin synthesis. These environmental stimuli include light, water, mineral, temperature, growth substances and pathogens (McClure 1986). Light induction is one of the rigorously studied environmental factors. Several observations in maize indicate that light affects the anthocyanin synthesis in different tissues. For example, irradiation of the coleoptile with UV-B (295 nm) for a few seconds is sufficient to induce anthocyanin synthesis (Beggs et al. 1985). If light is excluded from wild type ears (*C1, R1, Vp1*) during development, the ears produce colorless seed. However, exposure of these freshly harvested ears to light will induce pigment production (Cone et al. 1993a, Dooner and Ralston 1994).

Two alleles (*C1* and *c1-p*) at the *c* locus also reflect the light effect on the pigmentation. The *C1* allele (color determining) is the dominant wild type allele. When the anthocyaninless *vp1* mutant (*vp1, C1*) seeds are removed from the ear prior to desiccation and allowed to germinate under light, anthocyanin will appear in scutellum and aleurone layer after several days (Hattori et al. 1992). Another allele, called *c1-p*, is the so-called positive recessive allele. The *c1-p* allele can form anthocyanin under certain conditions in comparison to the *c1-n* (recessive negative) null allele which can not synthesize anthocyanin at any time. Seeds homozygous for the *c1-p* allele remain colorless throughout seed development. However, these colorless seeds will develop deep color in the aleurone if germinated in the light (Chen and Coe 1977).

Based on these observations, McCarty (1995) suggested that two independent mechanisms regulate anthocyanin synthesis in seed tissues. One is *C1* expression during seed maturation which requires VP1 function and therefore is coupled to the seed maturation program. The other is light activation during seed germination which is independent of VP1 function. However, these two different developmental mechanisms of tissue-specific activation of the anthocyanin pathway are intriguing because of the observation that *c1-p* seed can synthesize anthocyanin when germinated in the dark if they are exposed to light at some point during seed development (Chen and Coe 1977). Somehow the light signal in *c1-p* kernels is perceived and stored during seed development to be expressed only later during germination.

Based on the information that VP1 and ABA activate the C1-sh-GUS reporter gene in the protoplast electroporation assay (Hattori et al. 1992) and the fact that *C1* gene plays a critical role in the anthocyanin synthesis that is inducible by different environmental stresses, we reasoned that some of the environmental signals are likely to act through the *C1* gene at the transcriptional level. Following this idea, the purpose of this study is 1) to define the cis-elements for VP1 and ABA regulation in *C1* promoter, 2) to determine whether other factors (environmental or hormonal) can transcriptionally activate C1-sh-GUS expression, and 3) to examine the interaction of these factors.

Using protoplast electroporation and particle bombardment assays, it is demonstrated that the cis-elements for VP1 and ABA regulation overlap and that a 16 bp oligonucleotide dimer is sufficient for both VP1 and ABA activation. Of the environmental (light, cold, fungal elicitor and osmolarity) and hormonal (IAA and GA) factors we tested, only light has an effect on the *C1* promoter GUS gene. Light activation has a synergistic effect on VP1 and ABA regulation. Red, blue and ultraviolet light (UV) are effective, indicating that multiple photoreceptors are involved. Far-red light inhibited *C1* expression, suggesting possible involvement of phytochrome. The minimal light responsive cis-element is separate from the VP1/ABA response element and has features that resemble previously identified UV response sequences.

In addition to the VP1, ABA and light cis-elements, we identified a potential negative regulatory element which includes an ACGT motif. The light activation of C1-sh-GUS functions in different tissues including root and leaf tissues where *C1* is not normally expressed in vivo. However, the VP1, ABA and negative regulatory functions are specific to seed tissues.

# REVIEW OF LITERATURE

Anthocyanins are a class of phenolic compounds known as flavonoids which have a basic structure consisting of two aromatic rings joined by a three-carbon unit (C6-C3-C6). The three-carbon unit and ring B are derived from p-coumaric acid; the A ring is assembled from three molecules of malonyl CoA. Anthocyanins are water soluble and accumulate in the vacuole of plant cells. Anthocyanins may produce the red, violet or blue color in plant tissues depending on the number of hydroxyl groups on the B ring and the pH value of the cell (Goodwin and Mercer 1983). Because anthocyanins are not essential for plant survival and have a conspicuous phenotype, genes affecting synthesis of anthocyanin have been a favored subject for plant geneticists. Therefore, much information has accumulated since the garden pea pigmented flower phenotype was first studied by Gregor Mendel.

In maize, at least 20 genes involved in the synthesis and distribution of anthocyanins have been identified by mutation. These include structural genes, regulatory genes, modifiers as well as dominant inhibitors (Styles 1986). During seed development and maturation, anthocyanins accumulate in certain tissues of maize seed (the scutellum of embryo and the aleurone layer of endosperm). At least 8 loci (*Vp1*, *C1*, *R*, *C2*, *A1*, *A2*, *Bz1* and *Bz2*) are required for this pigmentation in the aleurone layer (Dooner 1983, 1985). In addition to the particular genetic requirements, the expression of anthocyanins in certain tissues occurs in response to complex developmental and environmental controls.

## Anthocyanin biosynthetic pathway

The anthocyanin biosynthetic pathway is well established in maize. The first reaction committed to the flavonoid biosynthesis is the condensation of three molecules of malonyl CoA and one of p-coumaroyl CoA to yield chalcone. This is catalysed by the enzyme chalcone synthase (CHS). Chalcone isomerase (CHI) catalyses the stereospecific isomerization of the chalcone to the colorless flavanone. Flavanone is converted to unpigmented dihydroflavonol by the action of flavanone 3-hydroxylase (F3H) at the C3 position. The dihydroflavonal is then reduced by dihydroflavonal 4-reductase (DFR) to yield a colorless leucoanthocyanidin that is in turn converted into a colored anthocyanidin by one or two uncharacterized enzymes. Anthocyanidin may be further modified by glycosylation which then produces the anthocyanin (Dooner 1985).

## Structural Genes of the Anthocyanin Pathway

Long before the first flavonoid pathway enzyme was detected, the maize geneticists had been trying to establish the order of gene action in the anthocyanin pathway. They used tools like epistatic interaction (Coe 1957) and inter tissue complementation (Reddy and Coe 1962). These results, together with the information collected by analysis of accumulated intermediates in specific genotypes, feeding with suspected precursors and by gene dosage effects on enzyme activity, led to the linear gene action sequence and the gene-enzyme relationships shown in Figure 1. Most of the structural genes of the anthocyanin pathway in maize have been isolated by transposon tagging (*C2*, *A1*, *A2*, *Bz1* and *Bz2*). The exception, *whp*, is a second CHS gene expressed in plant tissues but not in

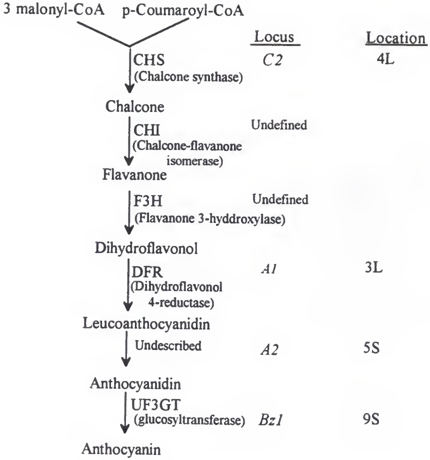

Figure 1. The simplified anthocyanin biosynthesis pathway, showing the enzymes for each step, the structural genes and their chromosomal locations. The genes for CHI and F3H are not identified in maize yet (modified from Dooner and Robbins 1991).

the aleurone. The *whp* gene was isolated by homology to *C2* gene (Dooner and Robbins 1991).

## Regulatory Genes of the Anthocyanin Pathway

In addition to the structural genes, a set of regulatory genes are well documented to govern the activity of the structural genes of the anthocyanin pathway. These regulatory genes were first noticed by the observation that they coordinately affect two or more unlinked structural genes of the anthocyanin biosynthetic pathway. For example, *c1* mutants have low levels of the *A1* and *Bz1* mRNA and enzyme activities (Cone et al. 1986, Dooner and Nelson 1977, Dooner 1983); and in *r* mutant aleurones, the mRNAs of *A1* and *C2* are not detectable (Dooner 1983, Ludwig et al. 1989). These regulatory genes in the anthocyanin biosynthesis pathway can be categorized into two groups: regulatory genes concerned only in the biosynthesis of anthocyanin pathway (i. e. *C1*, *Pl*, *R1* and *B*) and those that have pleiotropic effects (i. e. *Vp1*).

*C1*, *Pl*, *R1* and *B* regulatory genes have been grouped into two families, *C1/Pl* and *R/B*, based on the fact that the genes in each family encode the functionally equivalent proteins. Anthocyanin biosynthesis in any tissue requires the interaction between a member of each family. For example, for anthocyanin to be synthesized in the aleurone layer, a functional C1 and R1 protein have to be present; and Pl and B interact to specify pigmentation in the plant tissues. It is believed that *R* and *B*, *C1* and *Pl* are functional duplicated genes that have acquired different tissue specific expression patterns (Chandler et al. 1989, Cone et al. 1993a).

The phenotype of viviparous mutants, kernels that germinate while still on the ear, has been studied for many years (Robertson 1955). Several different loci have been identified including *vp1*, *vp2*, *vp5*, *vp7*, *vp8* and *vp9*. Most of these viviparous mutants cause reduced abscisic acid (ABA) levels in the seed. The *vp1* mutant is exceptional in that it accumulates wild-type levels of ABA, but has reduced sensitivity to that hormone.

In addition to the viviparous phenotype, the *vp1* mutant has unique pleiotropic effects on seed development. Most notable is that the anthocyanin biosynthesis pathway is blocked in the aleurone layer during seed development. Consistent with this colorless phenotype, the activities of the enzymes catalyzing anthocyanin biosynthesis were not detectable in the *vp1* mutant seed (Dooner 1985). *vp1* mutants fail to express the *C1* mRNA (McCarty et al. 1989). This observation suggests that *vp1* has a regulatory function in anthocyanin biosynthesis.

At least one allele from each regulatory gene of the anthocyanin pathway has been cloned and analyzed at the molecular level. Evidence from sequence analysis of the coding regions and functional assay of the encoded proteins in all cases support the regulatory roles played by these genes. In other words, the products encoded by these regulatory genes of anthocyanin pathway are all transcriptional factors.

*C1* regulatory gene. *C1* gene was cloned by Spm and En transposable element tagging in two laboratories at about the same time (Cone et al. 1986, Paz-Ares et al. 1986). The deduced amino acid sequence shows that *C1* encodes a protein of 273 amino acids with a molecular weight of 29 kD (Paz-Ares et al. 1987).

The C1 protein has sequences in amino-terminal end that are homologous to the DNA-binding domain of the *myb* oncogene. In addition, *C1* contains an acidic domain in the carboxy-terminal region with properties of a transcriptional domain of the protein. Further analysis of the protein by domain switching between C1 and GAL4 has confirmed that the C1 protein is a transcriptional activator with a functional DNA-binding domain and activation domain (Goff et al. 1991).

That a fusion protein consisting of the C1 DNA binding-domain and GAL4 activation-domain can complement the anthocyanin synthesis in *c1* mutant aleurone layer also suggests that the transcriptional activator activates the structural gene promoters of anthocyanin pathway. The cis-elements in the promoter of structural genes required for C1 regulation have been defined (Goff et al. 1991). The features of the C1 protein are summarized in Figure 2.

*R1* regulatory gene. The *R1* locus is one of the most complicated gene systems in the plant kingdom. *R1* locus has more than 100 different alleles that have been described with respect to the temporal and spatial regulation of anthocyanin biosynthesis (Coe et al. 1988). For anthocyanin pigmentation in any tissue of maize, a functional *R* gene product is only one of the important requirement.

*Lc*, a member of *R* gene family, which is responsible for pigmentation of midrib, ligule, auricle, glume, lemma, palea and pericarp tissues, was cloned and analyzed at the molecular level (Ludwig et al. 1989). The Lc protein deduced from cDNA sequence is composed of 610 amino acids. Within this 610 amino acids, there are distinctive features

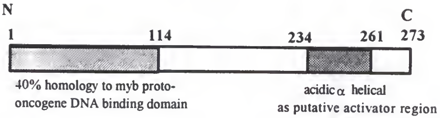
N
C
1
114
234
261
273
40% homology to myb proto-oncogene DNA binding domain
acidic α helical as putative activator region

Figure 2. Domain structure of C1 protein showing two domains with features of a transription factor. The location of putative DNA binding and activation domains are as indicated.

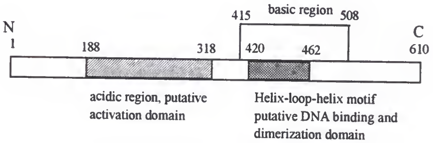
415
basic region
508
N
C
1
188
318
420
462
610
acidic region, putative activation domain
Helix-loop-helix motif putative DNA binding and dimerization domain

Figure 3. Proterin domain structure of one member of *R1* family ( *Lc*). Lc protein has features of the transcriptional activator as indicated. A seed specific member of *R1* family (*R-S*) has a similar structure.

of a DNA-binding domain and activation domain that are similar to other transcriptional activators. The putative activation domain and DNA-binding domain of Lc protein are located in an acidic region (amino acids 188 to 318) and a basic region (amino acids 415 to 508), respectively. A region within the basic domain of Lc (amino acid 420 to 462) has the helix-loop-helix structural motif conserved in *myc* oncogene that is proposed to function as a DNA binding and dimerization domain. The structural features of R1 protein (Lc) are summarized in Figure 3.

A cDNA clone from a seed-specific member of *R* gene family, *R-S* gene, has also been sequenced (Perrot and Cone 1989). At nucleic acid level, the *R-S* gene has 95% similarity to *Lc*. Most of the differences are distributed in the 5' untranslated region (Perrot and Cone 1989).

*Vp1* regulatory gene. The *vp1* mutant disrupts the normal embryo maturation program causing precocious germination on the ear prior to desiccation. In addition to vivipary, the *vp1* mutant fails to accumulate anthocyanin in the aleurone layer. The lack of anthocyanin pigment is due to a failure to express *C1* gene in the *vp1* mutant (McCarty et al. 1989). These pleiotropic effects suggest that the VP1 protein has at least three functions in wild type kernels: 1) to suppress the precocious germination of developing embryo and activate the seed maturation program, 2) to activate the anthocyanin biosynthetic pathway, and 3) to couple the anthocyanin pathway into the maturation program.

The *vp1* gene was cloned by transposon tagging and analyzed at the molecular level (McCarty et al. 1989a, 1991). It encodes a 2500 bp mRNA which is translated into a 73

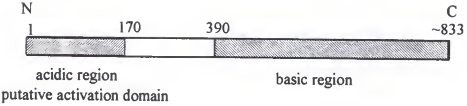

Figure 4. A simplified schematic figure of the VP1 protein. The VP1 protein has no significant homology to other transcriptional activators. The first 121 amino acids of VP1 have been shown act as an activator by domain-swapping experiments (modified from McCarty 1992).

kD protein. VP1 shows no sequence homology to any known proteins. There is no conserved DNA-binding domain or dimerization motif in the VP1 protein. However, the N-terminal region (amino acid 1 to 170) is highly negatively charged and a large positively charged domain is located at the C-terminal region. The domain structure of the VP1 protein is shown in Figure 4.

Although the VP1 protein has no conserved DNA-binding domain, at least 3 lines of evidence support the conclusion that VP1 is a transcriptional activator. First, a fusion protein containing the GAL4 DNA-binding domain (amino acid 1 to 147) and the VP1 acidic domain (amino acid 1-121) can activate a reporter gene with the GAL4 binding site in its promoter (McCarty et al. 1991). Second, over-expression of VP1 in maize protoplasts can transactivate the downstream *Em* gene promoter contained in Em-GUS reporter construct. Moreover, the N-terminal acidic domain in VP1 protein is critical for this activation (McCarty et al.1991). Finally, the activation of the *C1* promoter by over-expression of VP1 in protoplasts transformed by electroporation not only showed that the *Vp1* gene encodes a transcriptional activator, but also connects the function of *Vp1* gene to the regulatory cascade controlling anthocyanin biosynthesis in aleurone layer. A hierachical relationship between *VP1* and *C1* in the anthocyanin pathway is also supported by the fact that the colorless phenotype of the *vp1* mutant is associated with failure to express the *C1* gene in the kernel (McCarty et al. 1989).

## Alleles of *C1* Locus and their Phenotypes

There are at least 5 different phenotypically distinct alleles known at *C1* locus, color-determining (*C1*), dominant inhibitor (*C1-I*), super allele (*C1-S*), recessive positive (*c1-p*) and the null recessive allele (*c1-n*). The *C1* allele effect on anthocyanin expression in the triploid endosperm is dosage-dependent, i. e. *C1/C1/C1* and *C1/C1/c1* seed are purple, but *C1/c1/c1* seed are pale. The inhibitor allele, *C1-I*, is almost completely dominant over *C1*, i. e. *C1-I/C1/C1* are colorless (or a few purple dots). *C1-S* allele shows partial dominance over *C1-I*, i. e. *C1-S/C1-S/C1-I* results in colored aleurone, whereas *C1-S/C1-I/C1-I* is colorless (Coe et al. 1988, Cone et al. 1986). The *c1-p* is described as a positive recessive allele. This allele can be conditionally colored if the kernel is germinated under light. The *c1-n,* null recessive allele fails to develop pigment under any circumstances (Chen and Coe 1977). The phenotypic differences affecting anthocyanin synthesis during seed maturation and germination are summarized in Table I.

The *C1-I* allele was cloned by Paz-Ares et al. (1990). At the DNA sequence level, *C1-I* differs from wild-type *C1* by only 3 bp in the promoter region (up to -601 relative to transcription start site). The major transcript of *C1-I* encodes a 252 amino acid protein with a molecular weight of 27 kD, compared to a 273 amino acid, 29 kD protein for the wild-type *C1*. An 8 bp insertion at position 993 causes the *C1-I* transcript to prematurely terminate at position 1005. In addition, there are 32 single base (out of 778 bp) sequence differences between *C1-I* and *C1* in the protein coding region. Most of these differences are scattered over the carboxyl terminal half of the protein. In the carboxyl half (C terminal), the changes result in a loss of negative charge in the region representing the

| Allele Types | Endosperm genotype | Anthocyanin | |
|---|---|---|---|
| | | Maturation | Germination |
| *C1* | *C1/C1/C1* | +++ | |
| | *C1/C1/c1* | +++ | |
| | *C1/c1/c1* | + | |
| *C1-I* | *C1-I/C1-IC1-I* | - | - |
| | *C1-I/C1-I/C1* | - | - |
| | *C1-I/C1/C1* | +/- | - |
| *C1-S* | *C1-S/C1-S/C1-I* | +++ | |
| | *C1-S/C1-I/C1-I* | - | - |
| *c1-p* | | | + |
| *c1-n* | | | - |

Table I. The phenotypes of different alleles on *c* locus of maize. *C1*(color determining) has dosage effect on pigmentation. *C1-I* (inhibitor) shows dominant inhibition to*C1* coloring effect which can be partially relieved by *C1-S* (super) allele. *c1-p* (recessice positive) can induce anthocyanin during germination under light. *c1-n*(recessive negative) is the null allele. "+" means positively pigmented and "-" means there is no pigment synthesized.

functionally defined activation domain. In the amino (N terminal) half, the first 114 amino acids corresponding to the DNA binding domain remain intact. It is speculated that the C1-I protein retains the ability to bind DNA; however, it has lost the activation function (Paz-Ares et al. 1990). A similar situation was shown to occur in the GAL4 system in yeast, which converted the GAL4 activator into a transcriptional repressor (Hope et al. 1988).

Another interesting allele of *C1* locus is *c1-p*. It has been known for some time that although certain recessive *c* strains do not develop pigment at all, other homozygous *c* strains can develop intense pigment if germinated under light (Hsu 1970). It is clear now that the former phenotype is caused by the *c1-n* allele (null allele) which develops no pigment at any time, whereas the latter is the function of *c-p* allele. In 1977, Chen and Coe conducted experiments on the effects of light and developmental stages on *c1-p* kernels pigmentation. The results can be summaried as follows: 1) when exposed to light on the ear, *c1-p* kernel remain colorless, however, when light-exposed colorless kernels germinate in the dark they develop intense color; 2) the light stimulus is indispensible for pigmentation in *c1-p* tissue and is most effective during early stages of germination; 3) low energy (100 Watt) and brief illumination (1 hr) is enough to induce pigment synthesis and 4) completely de-embryonated kernels still have the light induction response.

The *c1-p* allele has been cloned and sequenced (Scheffeler et al. 1994). A sequence comparison revealed 29 bp differences in the protein coding region which cause 16 amino acid changes in the 273 amino acids protein. However, in the promoter region there is a single difference. The *c1-p* promoter has only one copy of a GTGTC motif at -151 relative to transcription start site in place of two copies in the wild-type *C1* promoter (Hattori et al. 1992, Scheffler et al. 1994). It is believed that the *c1-p* allele produces a protein functionally similar to wild type. So the question is how this 5 bp difference in the promoter region can cause the dramatic difference in phenotype between *C1* and *c1-p*. And even more intriguing is how the light signal is stored and expressed only at germination in the *c1-p* tissue. There is little evidence of how this storage mechanism might work. One confirmed observation is that the light induction and embryo germination can be separated because de-embryonated kernels still pigment (Chen and Coe 1977).

## VP1, ABA, Light and *C1* Interactions

Based on the phenotypes of different *C1* alleles, McCarty (1995) concluded that there may exist two different pathways to activate the anthocyanin biosynthesis in seeds. One is coupled with the maturation program. The other is a default pathway which is solely dependent on light activation. During normal seed development, anthocyanin expression is coupled with the maturation program in wild-type *C1* tissues provided all other factors are present. This implies that the anthocyanin pathway is triggered by maturation signals. Major signals are the VP1 factor and ABA. Transient assays showed that VP1 as well as ABA are important in regulation of *C1* promoter (Hattori et al. 1992). However, viviparous seed caused by ABA-deficient mutants of maize (e. g. *vp5*) are not deficient in anthocyanins (Robertson 1953). This contrasts to the viviparous, colorless phenotype caused by *vp1* mutant, which has no VP1 protein, but normal ABA levels. These results suggest that 1) VP1 is required for ABA activation of *C1* during seed development, and that 2) VP1 plays a key role in coupling the anthocyanin pathway into the maturation program.

In addition to VP1 and ABA, light is also necessary for anthocyanin synthesis during maturation because wild-type maize ears that develop in total darkness produce colorless seeds (Cone et al. 1993b, Dooner and Ralston 1994).

The default light activation pathway can be illustrated by the fact that the *C1 vp1* single mutant and *c1-p vp1* double mutant seeds which are colorless during seed maturation become pigmented during germination. This points out clearly that there is a light dependent induction mechanism for the anthocyanin pathway during germination which does not require VP1 and ABA activation. Furthermore, Scheffler et al. (1994) showed that *C1* mRNA is increased during germination in the light.

## Light Regulated Gene Expression

Light is involved in many aspects of plant life including seed germination, seedling development, flowering, seed development and senescence. Some of these responses involve gene regulation at the transcriptional level. At a minimum, for light to activate a specific light-mediated gene there must exist an interaction network composed of a photoreceptor to perceive the light stimulus, a signal transduction pathway from the receptor to the trans-factors (which may include both positive and negative components), and finally the target cis-elements on the promoter of light-mediated gene (which must already be in a competent chromatin structure). Although much effort has been put in, we still do not have a complete picture of this light activation network.

## Photoreceptors

Plants utilize at least three different classes of photoreceptors to detect their light environment: phytochromes (absorb primarily red light 600 nm ~ 700 nm and far red light > 700 nm), blue light receptors (400 nm ~ 500 nm) and UV light receptors (< 400 nm). Among them phytochrome is the best characterized, whereas the UV light receptor has not yet been isolated.

## Blue-light Receptor

Blue light can initiate several biological responses in higher plants which include phototropism, inhibition of hypocotyl elongation and stomatal opening (Deng 1994). The former two biological responses have been studied by analysis of mutants. A mutant with reduced blue light inhibition of hypocotyl elongation (*hy4*) has been identified in *Arabidopsis* and cloned recently by gene tagging (Ahmad and Cashmore 1993). The protein encoded by *hy4* gene has high homology to microbial DNA photolyases, a class of flavoproteins which catalyze blue light-dependent reactions. The blue light receptors have long been postulated to be flavinproteins (Galston 1949, 1950).

## Phytochrome

Phytochromes exist in two spectrally distinct photointerconvertible forms. Pr, the red-absorbing form, has maximum absorbance at 660 nm, and Pfr, the far red-absorbing form, has maximum absorbance at 730 nm. It is proposed that in vivo phytochromes are synthesized as Pr which is the biologically inactive form. Upon absorption of red light, Pr is photoconverted into Pfr and subsequently initiates the various phytochrome-mediated responses. The absorption of far red light converts Pfr back to Pr and thereby cancels the biological effects (Elich and Chory 1994).

The phytochrome holoprotein exists as a dimer of two identical subunits. Each monomer consists of an apoprotein of about 1100 amino acids with molecular weight of 120 kD and a linear tetrapyrrole chromophore covalently linked to a Cys residue in the N-terminal domain of the apoprotein (Vierstra 1993). A putative catalytic region located in the C-terminal half has weak sequence homology with the histidine kinase transmitter component of the bacterial two-component signal transduction system (Schneider-Pontsch 1992).

In *Arabidopsis*, which is in this respect the best characterized plant system, five different phytochrome genes have been identified - *PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE* (Sharrock and Quail 1989). They are separated into two classes according to their expression pattern and protein stabilities: PHYA--light labile phytochrome which predominates in etiolated (dark-grown) plants--and PHYB-E--light stable phytochrome which is predominant in light-grown plants.

## Mutants in Light Signal Transduction Pathway

After the photoreceptor is activated by a light signal, there presumably exists a signal transduction pathway(s) to transfer this stimulus (or even amplify it) to the target genes. However, 43 years after the discovery of phytochrome (Borthwick et al. 1952), the network cascade remains undefined. This difficulty may due to the fact that there exists a complex developmental control over the photoreceptor itself (Fluhr and Chua 1986) and different classes of photoreceptors may have overlapping functions (Marrs and Kaufman 1989). In addition, many plant growth and developmental responses regulated by light are also affected by other factors including environmental stress and hormones (Elich and Chory 1994).

Two methods have been used to dissect the transduction pathway from the photoreceptor to the regulated responses or gene expression. The first is to isolate the developmental mutants that affect the specific steps in the pathway. The other, which has only developed recently, is to microinject the putative intermediates and/or the agonists and antagonists into the mutant cells to complement the transduction pathway. Most of the genetic and biochemical information generated so far is related to the phytochrome-mediated responses.

### Genetic Analysis

Growing plants have distinct morphologies when growing under light or dark conditions. For example, dark-grown (etiolated) seedlings of dicotyledonous *Arabidopsis thaliana* have elongated hypocotyls, unopened apical hooks, small folded cotyledons and undeveloped chloroplasts (etioplasts). Conversely, light-grown seedlings have short hypocotyls, no apical hooks, and opened and enlarged cotyledons with developed chloroplasts (Deng 1994). These two distinct morphogenic pathways of seedling development in the presene or absence of light have been used to screen a number of light-regulated photomorphogenic mutants, especially in *Arabidopsis*. For example, many long hypocotyl (*hy*) mutants in *Arabidopsis* were selected by screening the seedling lacking the inhibition of hypocotyl elongation in different regimes of light (i. e. red, far-red and blue light).

Mutants in this light-regulated photomorphogenesis pathway can be separated into two groups. The first one is the loss-of-function mutants which block phytochrome-related responses. This group can be further separated into phytochrome-deficient and phytochrome-normal category. The phytochrome-deficient mutants can be further separated into chromophore mutant, red-light response mutants and far red light response mutants. The second class of mutants are the constitutive mutations which cause the light-regulated responses to be constitutively expressed even when the plants are grown in the dark. These were isolated by screening for the photomorphogenic responses in the absence of light and have been very useful in elucidating the transduction pathway.

## Loss-of-function Mutants

Phytochrome deficient mutants. The *hy1*, *hy2* and *hy6* mutants affect the chromophore biosynthesis steps. Because these mutants affect all phytochromes the phenotypes are the most severe. These mutations have normal levels of phytochrome apoprotein but lack detectable chromoprotein. By feeding the mutant plants with tetrapyrrole precursors, a wild type phenotype can be restored (Park and Quail 1991).

The *hy3* mutant, caused by a defect in the *PHYB* gene (Reed et al. 1993), is a red-light response mutant. The *hy3* mutant lacks most of the light stable phytochrome, but has normal levels of the light liable phytochrome A. The phenotype of this mutant includes accelerated flowering time, deficiency in chlorophyll accumulation, elongated stems, petioles and root hairs. Other phytochrome-mediated reponses affected include red light inhibition of hypocotyl elongation and the shade-avoidance response. Because these affected responses are all apparant in plants grown in light, phytochrome B has been proposed as one of the main photoreceptors that mediate the light response after the seedlings emerge from the soil (Vierstra 1993).

The *hy8* (or *fre1* for far red elongated) mutant which disrupts phytochrome A accumulation is a far red light response mutant (Whitelam et al. 1993, Nagatani et al. 1993, Park and Quail 1993). The *hy8* mutant have the reciprocal effect of the *hy3* mutant mentioned above. The mutant is deficient in light labile phytochrome A, but has normal levels of light stable phytochrome B. The *hy8* mutant seedlings show wild type responses to continuous red light and grow normally in white light. However, the far red light responses mediated by phytochrome A are lost. Under far red light, hypocotyls of *hy8* seedlings continue to elongate in contrast to wild type and *hy3*. Because the far red light inhibition of hypocotyl elongation is most apparent in etiolated plants and phytochrome A is predominant in dark-grown plants, phytochrome A is proposed to be the primary photoreceptor active before the seedling emerges from the soil. Once the seedlings reach above the soil, phytochrome A is converted into Pfr form which is rapidly degraded and further synthesis of phytochrome A is inhibited. Subsequently, the light stable phytochrome B is the predominate photoreceptor (Deng 1994, Vierstra 1993).

Phytochrome normal mutants. The *hy4*, *blu1*, *blu2* and *blu3* mutants reduce the inhibition of hypocotyl elongation in blue light. They have normal phytochrome levels and responses. The *hy4* gene was cloned by *Agrobacterium* T-DNA tagging. The gene was shown to encode a candidate blue-light photoreceptor protein with significant homology to microbial DNA photolyase which is a flavinprotein that catalyzes a blue-light dependent reaction (Ahmad and Cashmore 1993).

The *hy5* mutant seedlings do not have the red, far-red and blue light regulated inhibition of hypocotyl elongation. However, *hy5* seedlings have normal level of phytochromes. Therefore, the *hy5* gene may encode a component in the signal transduction pathway for both phytochrome and blue light mediated hypocotyl growth response.

Constitutive mutants. A total of 8 loci (3 *det* and 5 *cop*) have been identified in the constitutive response mutant category so far. They all are recessive, which suggests that the wild type gene products function to repress the photomorphogenesis processes in the dark. Light may release this repression so that the plants show the photomorphogenesis response in light. This repressor hypothesis is supported by evidence that over-expressing COP1 protein in transgenic *Arabidopsis* plants causes a dosage-dependant suppression of photomorphic seedling development under specific light conditions (McNellis et al. 1994).

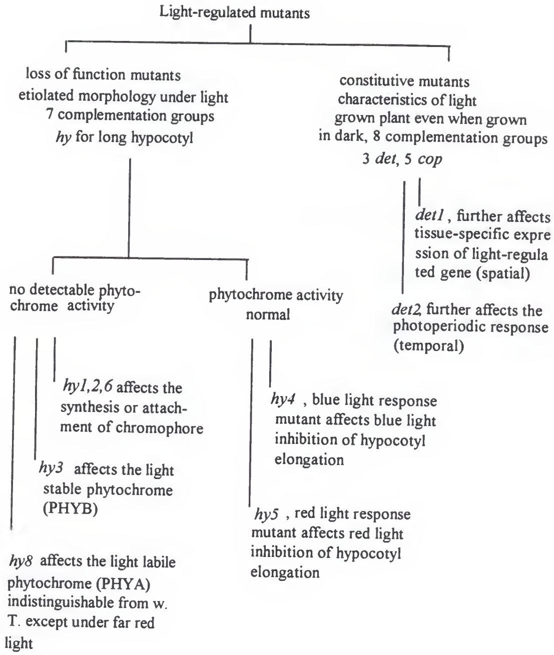

Figure 5. A summary of mutants affecting light-regulated seedling development in *Arabidopsis*. Not all of the identified mutants have been included in each class.

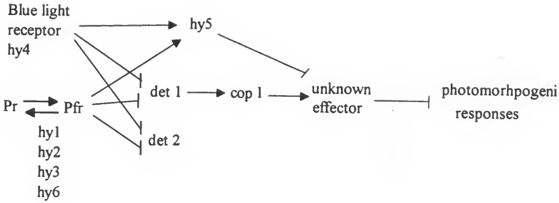

Figure 6. One possible gene action model for photomorphogenesis suggested by epistasis analysis. The lines with arrows indicate positive action or activation; the lines with "T" bars indicate negative action or repression, or not yet defined (modified from Chory 1992, Ang and Deng 1994).

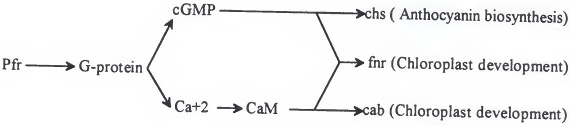
cGMP
chs ( Anthocyanin biosynthesis)
Pfr
G-protein
fnr (Chloroplast development)
Ca+2
CaM
cab (Chloroplast development)

Figure 7. Summary of phytochrome signal transduction pathways derived from microinjection assyes. *chs* (chalcone synthase gene) represents the anthocyanin pathway, *frn* (ferredoxin gene) and *cab* (cab-Gus marker) represent chloroplast development. he *chs, fnr*and *cab*genes in vivo have all been shown under phyotochrome regulation adapted from Neuhaus et al. 1993).

Among the constitutive mutants identified, the *cop1* gene has been cloned and molecularly characterized. It encodes a novel protein with a molecular weight of 74 kD which contains a putative $Zn^{+2}$-binding motif, a domain containing multiple WD40 $\alpha$-helix repeats and a region homologous to the $\beta$-subunit of trimeric G-protein (Deng et al. 1992, von Arnim and Deng 1993). Also it has been shown that COP1 protein reveals significant homology to the TAF 80 subunit of the TF IID complex of *Drosophila* (von Arnim and Deng 1994). All together, these results suggest that COP1 might have the ability to interact with the transcription machinery. In addition, it was shown that a fusion protein of COP1 and GUS is enriched in nucleus in darkness and in response to dark or light there exists accordingly a change in localization of nuclear GUS-COP1 fusion protein (von Arnim and Deng 1994). The COP1 sequence also has a putative nuclear localization signal (NLS) (McNellis et al. 1994). Together these results imply that COP1 protein can move between cytoplasm and nucleus and may act as a repressor within the nucleus to suppress photomorphogenesis processes by affecting gene expression. Therefore, COP1 protein may represent one of the final components in the transduction pathway which actually binds to and alters target gene expression. Another nuclear factor CA-1 from *Arabidopsis* was suggested to play a similar repressor role in *cab* gene regulation (Sun et al. 1993). In summary, these light-regulated mutants can be classified as shown in Figure 5.

The single mutant phenotypes tell us little about the functional relationships among these genes. Epistasis analysis is one approach to establish the order of gene action among a set of developmental genes without knowledge of molecular interactions. By comparing the phenotypes of double mutants to single mutations, the functional relationships among them can sometimes be inferred (Ambros 1989). For example, plants homozygous for *hy1* and *det1* exhibit the dark-grown and light-grown phenotypes of *det1*, suggesting that *det1* mutations are epistatic to the *hy1* mutations and that *det1* acts downstream of the *hy1* (Chory 1992). By this epistasis analysis, Chory (1992) and Ang and Deng (1994) established a genetic hiearchical relationship network for the photomorphogenic responses in *Arabidopsis*. The combined model is shown in Figure 6. Furthermore, these results suggested that 1) although light signals are perceived by different photoreceptors, the signals converge downstream and act through a common cascade of regulatory steps, and 2) photomorphogenic development of seedling is regulated through a series of de-repression circuits (Ang and Deng 1994).

### Biochemial Data from Microinjection Experiments

Various biochemical agents including calcium, phosphoinositides, cyclic AMP (cAMP) and acetylcholine that are known to act as second messenger signaling components in animal cells can also affect the phytochrome mediated responses such as protoplast swelling, chloroplast movement and fern spore germination (Tretyn et al. 1991). Although there has been much data accumulated in plant systems using these signaling agents, these studies have two inherent problems. First, the physiological responses like protoplast swelling and chloroplast movement are rapid and basically cytoplasmic events which may not involve gene regulation. Second, although many agonists and antagonists were defined for the responses, there is little known about the order of action (Bowler and Chua 1994).

Neuhaus et al. (1993) developed a single-cell microinjection assay which addressed these two concerns in a plant system.

They injected different putative signaling agents along with reporter gene construct (cab-GUS) into the tomato phytochrome mutant, *aurea,* which contains less than 5% of type I phytochrome (phytochrome A) in the dark (etiolated plants). Using this system they were able to work out the signal transduction pathways specific for the anthocyanin biosynthesis, chloroplast development and cab-GUS gene expression. They showed that a heterotrimeric G-protein acts as the most upstream component of the signal pathway for the anthocyanin biosynthesis, chloroplast development and cab-GUS gene expression. Downstream of the G-protein the transduction pathway is branched into cGMP-dependent and $Ca^{+2}/Ca^{+2}$ activated calmodulin ($Ca^{+2}$/CaM)-dependent pathways specify for anthocyanin biosynthesis and chloroplast development, respectively, as shown in Figure 7.

## Cis-acting Elements and Trans-factors for Light, ABA and Repression

Light-induced gene regulation has been studied using several different genes. Among them, the best characterized models are *cab* genes (chlorophyll a/b binding protein of photosystem II), *rbcS* genes (small subunit of RuBP carboxylase) and *chs* genes (chalcone synthase).

The light activation of *cab* and *rbcS* genes is phytochrome-mediated, whereas the light regulation of the *chs* gene in parsley has focused on UV light activation. It has been shown that blue light and phytochrome are also involved in regulation of *chs* gene expression (Bruns et al. 1986, Ohl et al. 1989, Smith et al. 1990, Feinbaum et al. 1991).

There are several reports of positive and negative blue light-mediated gene regulation, e. g., the pea *rbcS-3A*, *-3C* (Fluhr and Chua 1986), *cab* gene (Feinbaum et al. 1991) and cDNA clone pEA25 (Marrs and Kaufman 1989) are positively regulated and pea cDNA clone pEA207 (Marrs and Kaufman 1989) is negatively regulated. Some of these blue light-regulated gene promoters have been investigated to try to elucidate the blue light specific cis-elements and trans-factors. Although the sequences between -523 and -186 bp of *chs* gene from *Arabidopsis thaliana* were reported as necessary for blue light-induced expression (Feinbaum et al. 1991), thus far no blue light specific sequences or factors have been reported.

## Light Cis-element in *cab* Gene Promoter and their Trans-factors

The *cab* gene product, chlorophyll a/b binding protein, is the major protein component of the chloroplast thylakoid membrane which is mainly associated with photosystem II. CAB proteins bind noncovalently to chlorophyll a, b and carotenoid molecules where they function to harvest the light energy for photosynthesis (Leutwiler et al. 1986). These chloroplast thylakoid proteins are products of a small nuclear multigene family. CAB proteins are synthesized in the cytoplasm then transported into the chloroplast. The expression pattern of *cab* genes was shown to be transcriptionally regulated by light (phytochrome) and leaf-specific (Karlin-Neumann et al. 1988). Lamppa et al. (1985) showed that a chimeric gene containing a 400 bp fragment from pea *cab* gene promoter and neomycin phosphotransferase II (NPTII) coding region is regulated in a light-inducible and tissue-specific fashion in transgenic plants. Later it was shown that only

247 bp (-347 to -100) of this 400 bp fragment can perform this light-inducible and tissue-specific expression (Simpson et al. 1986). A similar situation was found in the wheat *cab-1* gene. A 268 bp enhancer-like element (from -357 to -89) is responsible for the phytochrome-mediated response of the gene (Nagy et al. 1987).

The *cab-1* gene in *Arabidopsis,* however has been studied more extensively. Ha and An (1988) generated and assayed a series of 5' deletion and internal deletion mutants of the *Arabidopsis cab-1* gene promoter. More than 20 of these mutants were assayed in transgenic tobacco plants. The results show that there are at least three cis-elements involved in light regulation. Two of these are immediately upstream (-253 to -158 , -321 to -253) and a third is located at further upstream (-1396 to -766). The region located between -253 and -158 was sufficient for promoter specificity; and the -321 to -253 element was required for maximum expression of the gene (Ha and An 1988).

Karlin-Neumann et al. (1988) further showed that constructs deleted to -183 were able to direct reporter expression in a phytochrome-mediated manner. There was no response at all for a -88 construct. These results demonstrated that sequence from -183 to -88 is necessary for phytochrome-regulated expression. Using this region as probe, the same group identified a *Arabidopsis* nuclear factor of about 70 kD, called CA-1 (for the region it binds has a high cytosine and adenine content), that specifically binds to the region. DNase I protection assays showed that the CA-1 factor protected a region (from -138 to -111, AACC*ACGT*AAGCAAACAAACAATCTAAA) of the promoter which includes a G-box motif that has been involved in many environmental and stress responsive cis-elements. The CA-1 factor itself does not show a light-inducible expression pattern.

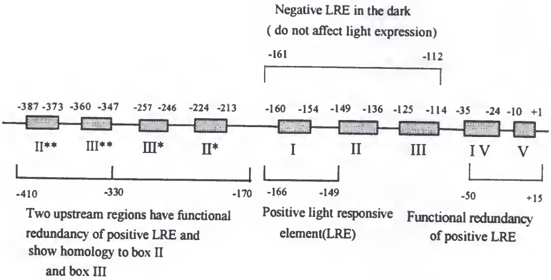
Negative LRE in the dark
( do not affect light expression)
-161
-112
-387 -373 -360 -347 -257 -246 -224 -213 -160 -154 -149 -136 -125 -114 -35 -24 -10 +1
II** III** III* II* I II III IV V
-410 -330 -170 -166 -149 -50 +15
Two upstream regions have functional redundancy of positive LRE and show homology to box II and box III
Positive light responsive element(LRE)
Functional redundancy of positive LRE

Figure 8. Upstream light responsive elements of pea *rbcS-3A* gene promoter. Boxes I to V are conserved among pea *rbcS* genes. Box IV is the TATA box. Boxes I, II, I II*, III*, II** and III** are GT-1 binding sites. The positive and negative LRE and functional redundancy for light are shown.

However, dephosphorylation of the CA-1 factor changed the mobility of the CA-1-DNA complex in the gel shift assay. In addition, CA-1 factor is not detected in *det1* mutant seedlings (Sun et al. 1993). Because the *cab-1* gene is constitutively expressed in *det1* mutant, the authors suggested that CA-1 may act as a transcriptional repressor in the wild type seedling (Sun et al. 1993).

### Light Cis-element for *rbcS* Gene and their Trans-factors

Ribulose-1,5-bisphosphate carboxylase catalyzes carboxylation and oxygenation of ribulose-1,5-bisphosphate which represents the first step in the photosynthetic Calvin cycle and photorespiration pathways, respectively. The holoenzyme of RuBP is composed of eight large subunits encoded and synthesized in the chloroplast and eight small subunits encoded by the nuclear genome and synthesized on cytoplasmic ribosomes. Like *cab*, the *rbcS* genes form a gene family and some of these genes including pea *rbcS-3A*, *-3C*, *-E9* and *ss3.6* have been shown to have organ-specific expression and to be transcriptionally regulated by light (phytochrome). In 1984, Herrera-Estrella et al. showed that 5' upstream region (-943 to -4) of pea *rbcS ss3.6* gene in either orientation conferred light inducibility to a heterologous chimeric reportor construct in the transformed tobacco calli. At about the same time, Morelli et al. (1985) used 5' flanking sequences of pea *rbcS-E9* to show that only 33 bp (from -35 to -2) is sufficient for light inducibility of this gene in the transformed petunia calli. The same group further demonstrated in transgenic tobacco and petunia that a 5' upstream fragment from -327 to -48 of the *rbcS-3A* is able to direct organ-specific and light-inducibility in either orientation. Similar results were obtained with the *rbcS-E9* 5' upstream region (from -317 to -82) (Fluhr et al. 1986). Further investigation of this 280 bp region from *rbcS-3A* gene showed that it is complex. This 280 bp sequence contained positive light responsive elements (LRE), a negative LRE and structural and functional redundancy which is summaried in Figure 8.

The next step is to characterize the trans-acting factors that interact with these elements. The pea nuclear extracts were used for gel-shift and DNase I footprinting assay with the different elements as a probe or binding templates. A factor from pea nuclear extracts that binds to the Box II (-151 GTGTGGTTAAATG -138), Box III (-125 ATCATTTTCACT -114), Box II* and Box III* was identified and called GT-1 (for the BoxII and III contain highly conserved GT motif) (Green et al. 1987). Later, Gilmartin et al. (1992) isolated a cDNA clone from etiolated tobacco cDNA library which encoded a DNA binding protein, GT-1a, with specificity for Box II sequence. The predicted structure of GT-1a (43 kD) has a putative helix-helix-turn-helix (HHTH) motif which may be involved in DNA binding. However, the GT-1a mRNA is present in several tissues, including both light and dark-adapted leaves and the binding activity is not light regulated. This suggests that if GT-1a factor is involved in this transcriptional regulation of *rbcS-3A* gene, there must be other factors or mechanisms that interact with or modifiy GT-1a in a light-mediated manner to confer the observed light responsive property of this gene (Gilmartin et al. 1992).

The apparent functional redundancy of upstream elements of the pea *rbcS-3A* gene mentioned above suggests that no single binding site is crucial for the function of the full promoter. This point argues the possibility that there may exist additional protein binding

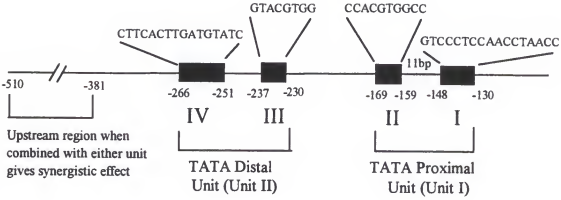
GTACGTGG
CCACGTGGCC
CTTCACTTGATGTATC
GTCCCTCCAACCTAACC
11bp
-510
-381
-266
-251
-237
-230
-169
-159
-148
-130
IV
III
II
I
Upstream region when combined with either unit gives synergistic effect
TATA Distal Unit (Unit II)
TATA Proximal Unit (Unit I)

Figure 9. Upstream light responsive cis-elements in *chs* promoter of parsley. Boxes I to IV are identified by in vivo footprinting. The TATA proximal unit was shown to to confer light responsiveness on 35S (-46) promoter. The 11 bp spacing in the Unit I is critical for light activation. One of the boxes (Box II and III) in each unit covers the G-box-like motif that is highly conserved among other light- and stress-induced plant genes (modified from Block et al. 1990).

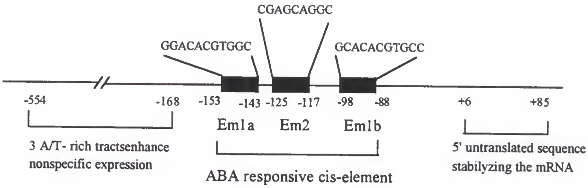
CGAGCAGGC
GGACACGTGGC
GCACACGTGCC
-554
-168
-153
-143
-125
-117
-98
-88
+6
+85
Em1a
Em2
Em1b
3 A/T- rich tractsenhance nonspecific expression
ABA responsive cis-element
5' untranslated sequence stabilyzing the mRNA

Figure 10. Summary of the ABA responsive functional domains in *Em* promoter of wheat. The Em1a and 1b cis-elements contain the G-box-like motif (ACGT motif) (modified from Guiltinan et al. 1990).

sequences which are required for *rbcS* gene transcription. In 1988, Giuliano et al. used nuclear extracts from tomato and *Arabidopsis* seedlings and various probes spanning from -1000 to -31 of the tomato *rbcS-3A* gene in the gel-shift assay and DNase I footprinting experiments. They identified a protein factor from tomato and *Arabidopsis* which specifically binds to a short conserved sequence (5'-TCTTAC*ACGT*GGCAYY-3' (where Y is pyrimidine), which includes a so called G-box motif (CCACGTGG) (Giuliano et al. 1988). The protein factors were cloned from *Arabidopsis* cDNA library and called GBF1, 2 and 3. The deduced amino acid sequences of these three cDNA clones indicate that they belong to the basic leucine zipper protein (bZip) class (Schindler et al. 1992a). Furthermore, homodimers and heterodimers among these GBFs also interact with the G-box (Armstrong et al. 1992, Schindler et al. 1992b). The proline-rich sequences at the N-terminus of GBF1 when fused to a heterologous DNA binding domain (e. g. yeast GAL4 amino acid 1 to 147) stimulated transcription in both plant protoplasts and mammalian cells (Schindler et al. 1992b).

### Ligth Cis-element for *chs* Gene and its Trans-factors

Another light responsive gene with extensive research history is the chalcone synthase gene (*chs*) of parsley (*Petroselinum crispum*). Chalcone synthase is the first committed enzyme in the flavonoid-specific branch of the phenylpropanoid pathway which leads to the biosynthesis of anthocyanin (Hahlbrock and Grisebach 1979). Krenzaler et al. (1983) first recognized that treatment of dark-grown parsley suspension cells with UV-containing white light induced accumulation of *chs* mRNA and flavonoids in vacuole. Unlike the gene families of the *rbcS* and *cab* genes, the *chs* gene in parsley is present in one copy per hapoid genome (Herrman et al. 1988).

The functional architecture of the light-responsive chalcone synthase promoter from parsley has been extensively analyzed using a protoplast electroporation assay. Basically the chalcone synthase promoter contains two separable cis-acting units that differ in strength. Each unit contains two short boxes (the TATA proximal unit I, has Box I and II; the TATA distal unit II has Box III and IV) identified by footprinting. One box in each unit (Box II, CCACGTGG and Box III, GTACGTGG) contains a G-box-like sequence. Box II and III are functionally interchangable and operate in an orientation-dependent manner (Block et al. 1990). However, the spacing between cis-elements had different effects in the two units. The activity of TATA proximal unit required Box I and Box II at a defined distance (11 bp apart). No rigid distance requirement was observed between Box II and III (Block et al. 1990). In addition, a third upstream region (from -510 to -381) had a synergistic quantitative role on the light activation of the promoter (Schulze-Lefert et al. 1989). The architecture of the light-responsive chalcone synthase promoter is summarized in Figure 9.

The sufficiency for light responsivness of the TATA proximal unit (52 bp) was proved by gain- of-function experiments in which the unit was put in front of a 35S minimal TATA box (-46) (Weisshaar et al. 1991). The putative trans-factors, CPRF-1, -2 and -3, which bind specifically to Box II were cloned from parsley cDNA library. The deduced amino acid sequences of all three proteins reveal the characteristics of bZip class transcriptional factors. Also mRNA accumulation of CPRF-1, but not CPRF-2 or

CPRF-3, is light-induced suggesting that CPRF-1 participates in the light-mediated activation of *chs* gene in parsley (Weisshaar et al. 1991).

## ABA Responsive Cis-element and their Trans-factors

The plant hormone abscisic acid (ABA) has been implicated in a number of important developmental and physiological processes including embryo maturation and the responses of vegetative tissues to osmotic stress. Hetherington and Quatrano (1991) classified the responses to ABA on the basis of whether the responses are rapid or slow. The ABA induced rapid responses involving ion fluxes (calcium ion) are typified by rapid stomatal responses. The slower responses, typified by the response of cereal embryos to ABA, require alteration of gene expression which involves cis-acting elements and trans-factors that regulate ABA-responsive genes (Hetherington and Quatrano 1991).

The ABA-responsive genes are mainly identified by the observation that during mid to late stage of seed development, these mRNAs accumulate in embryos in response to high endogenous ABA levels. If earlier stage embryos are isolated and exposed to exogenous ABA, some of the same mRNAs are precociously induced. Using these criteria several genes have been classified as ABA-responsive genes. These include a class of genes that encode proteins called LEAs (late embryo abundant proteins). Of them the wheat *Em* gene has been studied in great detail.

In 1988, Marcotte et al. showed that a chimeric gene with 646 bp (-554 to +92) of the wheat *Em* promoter is sufficient for ABA-regulated expression in a rice protoplast transient assay. The same group determined which part of the 646 bp promoter region is responsible for the ABA response. They showed that a -168 to +92 reporter construct gave 20-fold activation, whereas the -101 to +92 region gave only 3-fold induction in the presence of ABA. To further confirm this , a 50 bp sequence (-152 to -103) was fused to minimal 35S CaMV (-90) promoter. This reporter construct confers about 3-fold ABA responsiveness. A longer fragment from the same region (75 bp including Em1 and Em2, from -153 to -88 ) confers 11-fold ABA induction (Guiltinan et al. 1990). The sequences of Em 1 and Em 2 in the 75 bp region are conserved in other seed/ABA-responsive genes, for example in the ABA responsive element of the rice *RAB 16A* promoter (Mundy et al. 1990). Marcotte et al. (1988) also observed that 5' untranslated sequence (from +6 to +85) of the *Em* when inserted between the 35S promoter and GUS produced a 10-fold enhancement of GUS expression in the absence of ABA. However, in the -103 Em construct (-103 to +92), this element did not enhance expression. The authors suggested that this 5' untranslated region does not contain an enhancer function, but plays a role in stabilizing or increasing efficiency of translation of the mRNA (Marcotte et al. 1989). The cis-elements of ABA-responsive *Em* gene from wheat are summaried in Figure 10.

Guiltinan et al. (1990) reported a cDNA clone that encoded a protein (EmBP-1) with the binding specificity for Em1a (CACGTGGC). The deduced amino acid sequence of EmBP-1 contains a basic leucine zipper domain (bZip). Interestingly, EmBP-1 only interacts with the Em1a sequences (GGAACGTGGC), not with Em2 or Em1b (GCCACGTGCC), which differ from Em1a by only two base pairs (Guiltinan et al. 1990).

## Negative Regulatory Elements

Although it has been recognized that the negative regulation of gene expression may be at least as important as positive regulation, negative regulatory elements identified in plants are few and less well defined. In 1986, Simpson et al. first reported that a 247 bp element from *cab AB8.0* gene acts not only as a light-inducible enhancer but also as a tissue-specific silencer. Tissue-specific silencing was shown by placement of the 247 bp element in front of a constitutively expressed reporter construct (nos-NPT II). The expression pattern changed from a similar level in both leaf and root tissue to strong expression in leaf but no expression in root tissue. The authors suggested that the silencing effect in root is due to a repressor binding to this element. This repressor becomes inactivated in photosynthetic tissue (leaf), allowing a second positively acting factor to act on the upstream region (Simpson et al. 1986).

A similar situation has been reported in another light responsive gene, *rbcS-3A* gene from pea. A 58 bp element (include Box I, II and III) from pea *rbcS-3A* gene has a negative influence on the steady-state mRNA in dark-adapted plants (Kuhlemeier et al. 1987). In contrast to the *cab AB8.0* gene, the 58 bp sequence from *rbcS-3A* gene was inserted between 35S gene enhancer (-943 to -31) and TATA (-31 to +6) in a 35S-CAT reporter construct. In another case, a 5' deletion of tobacco *cab-E* gene (from -1182 to -747) caused a 5-fold activation of the chimeric CAT activity in transgenic plants (Castresana et al. 1988). None of the cognate trans-factors for these negative cis-acting elements have been identified.

From the above examples, several common features can be discerned:

(1) There is often a quantitative enhancer which gives the maximum expression and critical cis-elements which confer signal or tissue-specific expression in the 5' promoter region.

(2) The qualitative cis-elements are located within 200 bp relative to the transcription start site.

(3) The G-box motif is involved in many different cis-elements that respond to various stimuli and the cloned G-box binding factors belong to the bZip protein family.

(4) The cis-elements in a specific gene often show functional redundancy.

(5) In only one case (CPRF-1), does the putative cognate trans-factor show inducible expression. There is a second as yet identified G-box binding activity which exhibits light-regulated modification and nuclear translocation from cytosol (Harter et al. 1994).

(6) Negative elements are less defined and characterized.

(7) The cis-elements can function in heterologous system including a heterologous TATA and transcription start site (spacing between them seems not critical).

# MATERIALS AND METHODS

## Protoplast Electroporation

Protoplasts were prepared from an embryo-derived suspension cell line (Dekalb XL80 hybrid) 4- or 5-days after transfer to fresh media as described previously (Vasil et al. 1989). Effector (35S-sh-Vp1) and/or reporter (C1-sh-GUS) plasmid DNA (20 µg per 6 x 10 $^6$ protoplasts) was transferred into freshly isolated protoplasts using a Cell-Porator (BRL) apparatus at 180 v, 800 mu. Each experiment was replicated in triplicate. After electroporation the protoplasts were cultured in modified KM growth media for 40 to 45 hr. (Vasil et al. 1989).

## Particle Bombardment

The preparation of the plasmid DNA/gold mixture as well as the bombardment parameters were modified from the methods described previously (Taylor and Vasil 1991). Briefly, 37 µl of a 40 mg/ml gold stock solution (1.6 µm diameter, Biorad Inc.) was mixed in order with 25 ul water, 2 µg effector DNA, 2 µg reporter DNA and 5 µg internal control plasmid DNA in a 250 µl Eppendorf tube. The tubes were vortexed briefly before and after each addition. 20 µl of 100 mM free base spermidine and 50 µl of 2.5 M $CaCl_2$ were placed in separate drops on the side of the tube to avoid premature mixing of either solution with the DNA/gold solution. The tubes were then immediately mixed by vortexing for 10 s. At that time the plasmid DNA was precipitated and attached to the gold particle. The tubes were centrifuged for 5 s and the supernatant fluid removed. 200 µl of 100% ethanol were added and sonicated briefly. After brief centrifugation to pellet the gold/DNA, the supernatant fluid was removed. 100 µl of 100% ethanol were added and the tube placed on ice. A 5 µl volume of sonicated gold/DNA mixture was used for each bombardment shot. The different light wavelength treatments were obtained by placing wavelength cutoff filters (gelatin filters, Kodak) on the petri dishes and completely wrapping the dishes with alumium foil to ensure that light was admitted only through the filter. Each experiment was replicated at least in triplicate. The material was harvested after 40 hrs for ABA and VP1 treatments and 24 hrs for light treatment. The glucuronidase (GUS) enzyme activity of the reporter and luciferase (LUX) enzyme activity of the internal control were assayed as described below.

## Tissue Preparation

Developing ears harvested 17 to 22 days after pollination (DAP) and mature dry seed were surface sterilized by imersion for 1 min in 70 % (v/v) ethanol, 5 min in 10 % (v/v) commercial Clorox followed by two rinses with sterile water. The mature dry seeds were then imbibed in dark for 48 hrs to initiate germination and soften the tissue. The developing and germinating embryos were then removed from the kernel and placed on a gelrite solidified MS medium (Sigma, M5519) containing 20 g/l sucrose. Embryos were placed in a dark box prior to bombardment.

For particle bombardment of leaf tissues, leaves were harvested from seedling grown aseptically in magenta boxes (Sigma) under a 16/8 hr photoperiod. The youngest fully expanded leaves were cut into ~ 1 $cm^2$ sections and placed with the adaxial surface facing up on MS media containing 20 g/l sucrose. After bombardment the leaf segments were cut into half and put in separate petri dishes containing sterile water for dark and light treatment. Light and dark treatment were imposed as described above.

### Extraction and Quantification of Transient Expression

For protoplast electroporation experiments, following 40 hr of incubation the protoplasts were pipetted into a sterile tube and centrifuged in a DYNAC clinical centrifuge at lowest speed setting for 3 min. After removal of supernatant fluid, 2 ml of ice cold extraction buffer (0.1 M potassium phosphate pH 7.8, 2 mM EDTA pH8.0, 2 mM DTT, 5% glycerol) was added and mixed gently with a glass Pasteur pipette to disperse the cell pellet. Protoplasts were again precipitated by centrifugation, resuspended in 200 µl of extraction buffer, then ground by mortar and pestle with ca. 0.1 g of silicon carbide powder. The homogenate was mixed by vortexing 10 s and centrifuged to recover the supernatant cell extract. For particle bombardment, embryo or leaf tissues were ground individually using the same method.

GUS activities were assayed using the method previously described by Jefferson et al. (1987). For luciferase activities, 20 µl of the cell extract were mixed with 200 µl of ice cold reaction buffer (25 mM tricine pH 7.8, 15 mM MgCl2, 5 mM ATP, 0.05% BSA) in a reaction tube (12 x 75 mm) and assayed using a Monolight Luminometer (Analytical

Luminescence Laboratory). With the insertion of the tube into the luminometer, the machine automatically injected 100 μl of subtrate buffer (1 mM luciferin, pH 9.5). The emitted photons were counted for 15 s. Activities were recorded as standard Relative Light Units (RLU).

## Plasmid Constructions

Construction of the D00, D20, D30, D53, D54, D5354, M53, Id5, Id6, 8283, 8081, 7879, 3274 and 7677 plasmids were described previously (Hattori et al. 1992). To construct the multiple-base-change mutants 112, 118, 138, 120, 134, 128 and 126, the fragment EcoR I - Xba I of D29 was cloned into the pSelect vector (Promega) for directed mutagenesis using the Altered Site In Vitro Mutagenisis System (Promega) to generate the site-directed mutants. The synthetic oligonucleotides used to generate each mutant were as follows: mutant 112, 5'-CCCATGACAGATCTGTCGTGTCG-3'; for mutant 118, 5'-ATGACAGCAGCTGC- GTGTCGTCC-3'; mutant 138, 5'-CATGACAGCAGTGTTAACTCGTCCATGCATGC-3'; mutant 120, 5'-AGTGTCGTGTTAACCATGCATGC-3'; mutant 134, 5'-GTGTCGTGTCGTCGACGC- ATGCACTTTA-3'; mutant 128, 5'-TGTCGTCCATTACGGCACTTTAGG-3'; mutant 126, 5'-GTCCATGCATTACCTTTAGGTGC-3'. To construct the Sph20, the following complementary pair of oligonucleotides were used: 5'-GATCCGTGTCGTCCATGCAT-3' and 5'-GATCATGCATGGACGACACG-3'. The oligonucleotides were phosphorylated with T4 polynucleotide kinase and annealed. The compatible BamHI sites on the ends of this annealed pair allowed it to be cloned into pSelect at the BamHI site. A HindIII-EcoRI fragment excised from the Sph20 pSelect plasmid was ligated to the HindIII-EcoRI backbone of the CaMV 35S (-46)-GUS plasmid. The same procedure was used for construction of CLE 40, CLE 04, CLE 41, TCLE and SCLE. For CLE 40, CLE 04 and CLE 41 the complementary pair of oligonucleotides are 5'-GATCTGCTAACTACGTGGCCGAG-3' and 5'-GATCCTCGGCCACGTAGTTAGCA-3'. For the TCLE construct, the complementary pair was 5'-GATCCGGCCTCAACTCGGCCA-3' and 5'-GATCTGGCCGAGTTGAGGCCG-3'. For the SCLE construct, the complementary pair was 5'-GATCCGGGCCTCAACTCGGCCACGTAGTTAGA-3' and 5'-GATCTCTAACTACGTGGCCGAGTTGAGGCCCG-3'. To construct the LCLE, a 80 bp Nae I - Sph I fragment was excised from D29 and cloned into pSelect. The 124 bp Hind III - EcoR I fragment was excised from pSelect and ligated to the HindIII-EcoRI backbone of CHS1 plasmid in order to place it upstream of 35S (-46) minimal promoter. To make the D40 + SCLE, the 75 bp Sal I fragment was taken from SCLE and ligated into Sal I digested D40. To construct the negative mutant constructs NE 10 and NE 01, the 35S promoter fragment was excised from pBI 221 (HindIII - BamHI fragment) and ligated into pBluescript. Then the 31bp NaeI - DraI fragment from D29 was ligated into the EcoRV site of 35S promoter in pBluescript. Finally, the HindIII - BamHI fragment was excised from pBluescript and ligated back into pBI 221. To construct D30dNS, the 1145 bp SphI-EcoRI fragment from D00dNS was cloned into pSelect. Then the 345 bp HindIII fragment from the pSelect was ligated into the HindIII digested D00 backbone.

The same procedure was used for constructing the D10dNS, D20dNS and D29dNS. For D3035S construct, the synthetic complementary oligonucleotides including the 35S TATA region, the transcription start site and with 5' SalI site and 3' XbaI site compatible sequence were used. After the oligonucleotides were phosphorylated and annealed, the annealed pair was ligated into the SalI-XbaI backbone fragment of D30. To construct D3035SNI, the 80 bp SphI-NaeI fragment from D29 was cloned into pSelect, and the HindIII-EcoRI fragment from pSelect into CaMV 35S (-46) minium promoter. For the pl construct, the 500 bp comparable region of *pl* promoter (from XbaI to SalI ) was cut from pl-McC genomic clone (kindly provided by Dr. K. Cone, Univerity of Missouri) and cloned into pSelect. Then the EcoRI - SalI fragment was excised and ligated into EcoRI-SalI digested D00 background. All these plasmid constructs were sequenced to make sure the sequences were changed.

# RESULTS

## Dissection of the VP1 and ABA Response Element

The previous studies showed that a *C1* promoter GUS reporter gene (C1-sh-GUS) can be activated by over-expression of VP1 (35S-sh-Vp1) and ABA treatment in protoplasts transformed by electroporation. The sequences necessary for VP1 and ABA responses are located between -156 and -125 in the *C1* promoter (Hattori et al. 1992). As shown in Figure 11A, a series of site-directed mutants through this region were made and tested for their response in the protoplast electroporation assay. Figure 11B and C show the responses of these mutants to VP1 and ABA regulation, respectively. The results show that the sequence from -142 to -132 (5'-CGTCCATGCAT-3'), which is basically the Sph element , is critical for VP1 activation, whereas the sequence essential for ABA regulation extends 5 bp further 5' end to include residue -147. The region required for both ABA and VP1 activation is 16 bp long from -147 to -132 (5'-CGTGTCGTCCATGCAT-3').

We further tested this 16 bp element by cloning a synthetic oligomer of this sequence (CGTGTCGTCCATGCAT) upstream of the CaMV 35S minimal promoter (-46) and assaying this heterologous reporter construct by protoplast electroporation and particle bombardment of developing embryos. As shown in Figure 12, a dimer of this sequence (Sph 20) is sufficient for both ABA and VP1 activation, whereas no activation of monomer in either orientation (Sph 10 and Sph 01) was detected.

## Transient Expression Proves that *C1* Promoter can be Activated by Light

Light effects on anthocyanin biosynthesis have been implicated in many plant systems including maize. As shown in Figure 13, light can activate pigment synthesis in *vp1* mutant seeds. This and other observations (see Literature Review) imply that light can activate the *C1* gene and/or other genes in the pathway (for example, the *C2* chalcone synthase gene) in the absence of VP1. We first tested light activation using protoplasts from a dark-adapted suspension cell line which originated from maize embryo tissue and subsequently by particle bombardment of leaves and embryos.

Both transient expression systems showed that light alone can activate the full length *C1* promoter reporter construct (C1-sh-Gus) (Figures 14, 18, 19) and that this light activation effect is specific to C1-sh-GUS reporter construct, and not the other promoters tested (Figures 15, 30). Among the negative controls, C25 (ubiquitin promoter-*ubi* intron 1-GUS), Sh1 (*sh1* promoter-*sh1* intron1-GUS) and Sh2 (*sh2* promoter-*sh1* intron 1-GUS) include the first intron of the maize *shrunken* gene as in C1-sh-GUS. These results clearly indicated that the light activation is specific to C1-sh-GUS and is generated through interaction with the *C1* promoter region and not the *sh1* intron. From Figure 14, it is also clear that the plant hormone gibberellic acid (GA) which is an important regulator of seed germination has no effect on the *C1* promoter or the light effect.

We then postulated that there may exist light cis-element(s) that either overlap or act independently of the cis-element for VP1/ABA regulation within the *C1* promoter. Before further analysis, we first sought to optimize the expression system for light activation.

## Optimization of Conditions for the Light Activation Assay

In the particle bombardment assay, several factors can influence the expression of a reporter gene delivered into the target tissues (King and Kasha 1994, Kless et al. 1992, Vain et al. 1993). Some of these factors (for example, gold particle size, pressure limit of rupture disk, gap distance, fly distance, treatment of the material and the preparation of gold-DNA mixture) can be compensated and controlled by the use of an internal control plasmid (ubiquitin-sh-LUX) that is included in the gold-DNA mixture with the reporter construct. However, there are other factors that can qualitatively influence reporter expression. We conducted the following experiments to optimize the transient expression system.

Concentration of reporter DNA (C1-sh-GUS): As shown in Figure 16, in the range of concentrations tested (2, 5 and 10 μg DNA of C1-sh-GUS), an inhibitory effect was exerted by higher amounts of reporter DNA. The light effect is totally abolished at 10 μg DNA; 2 μg DNA was optimal for light activation. The suppression effect by higher amounts of reporter DNA may due to titration of the components of initiation complex or the activators (Radicella et al. 1992).

Different aged leaves and embryos had different sensitivities to light activation of C1-sh-GUS. As shown in Figure 17, samples from the upper and lower (older) leaves of the same seedling while showing similar expression levels of the internal control (LUX values), lost the light response (GUS expression). A similar situation occurs in older embryos; embryos at 27 DAP (in summer field) lost the ability to respond to light (Figure 17).

Optimal incubation time for C1-sh-GUS light activation: Figure 19 shows the time course for light activation of C1-sh-GUS in the protoplast and leaf particle bombardment assays. In protoplast electroporation experiments, at least 9 hrs of continuous illumination were necessary for significant activation. However, in leaf bombardments (from light-adapted seedlings) the light induction is detected after 6 hrs treatment and reached the maximum at about 24 hrs.

Based on this information, we established the following standard conditions for a light activation assay of C1-sh-GUS using the Helium gun system: a pressure limit of 1100 psi, 2 μg reporter DNA, 5 μg of internal control DNA (ubiquitin-LUX) and embryos at around 20 DAP or the latest fully expanded leaves of young seedling followed by a light treatment for 24 hrs.

## Light Activation has a Synergestic Effect on VP1 and ABA Activation

VP1 over-expression and the hormone ABA have been shown to activate the C1-sh-GUS reporter construct in maize protoplasts (Figures 11, 12, and Hattori et al. 1992). This suggested a possibility that light activation may act through VP1 or ABA to have an effect on *C1* activation. However, Figure 9 shows that the light activation of C1-sh-GUS can occur in embryos from both wild type and *vp1* mutant genotypes. The similar result derived from the experiment showing that the D30 construct (-130), which does not to repond to VP1 and ABA, still has strong activation by light (Figure 21).

In addition, the internal deletion mutants Id4 (deletion from -151 to -147) and Id5 (deletion from -136 to -131), which lack the critical sequences for ABA and VP1 activation respectively, still retain the light responsive (Figure 22). These results are consistent with the phenotypes summarized in Figure 13 and suggest that VP1 is not required for the light induction of anthocyanin synthesis. In summary, 1) the cis-element for light activation is separable from the cis-element for VP1 and ABA, and that 2) the VP1 and ABA functions are not required for light activation of *C1* promoter, which supports the proposal of two different mechanisms for anthocyanin induction in seeds (McCarty 1995).

Although light activation is separable from VP1 induction of *C1* promoter, there is an interaction between these factors. As shown in Figure 10, the combinational effects of light plus VP1 and light plus ABA activation are roughly multiplicative with respect to individual treatments. This positively synergistic relationship indicates that there exists an enhancing interaction between light, VP1 and ABA activation. This is consistent with the observation that these signals function synergistically in normal seed development. For example, wild type seeds grown in total darkness fail to accumulate anthocyanins even though VP1, *C1* and ABA are all present. Therefore a light signal would seem to be required in addition to VP1 and ABA signals for induction of the pathway during seed maturation. Conversely, the phenotypes of the *c1-p* allele and *vp1* mutants indicate that light alone is insufficient to activate *C1* in the absence of a functional ABA response element and the VP1 factor, respectively. These interactions suggest that the combinatorial arrangement of ABA, VP1 and light response elements in the *C1* promoter has a role in the integration of these signals.

## Both Phytochrome and UV Light Involved in *C1* Light Response

As mentioned earlier in Literature Review plants utilize at least three types of photoreceptors to detect environmental light signal: these include phytochrome, blue/(UV-A) and UV-B receptors. By using different wavelength cutoff filters, we were able to determine that UV, red light (> 600 nm ) and blue light (400 ~ 500 nm) positively affect C1-sh-GUS, whereas far red light (> 700 nm) was inhibitory (Figure 23). This suggests that the light activation in this case involves multiple photoreceptors and phytochrome may be one of them. The expression of *rbcS* gene in green tissue (but not etiolated tissue) requires action of both phytochrome and a blue receptor (Fluhr and Chua 1986). The expression of parsley *chs* gene is also under the dual control of a UV light photoreceptor and phytochrome (Drumm and Mohr 1974). Yatsuhashi et al. (1982), and Oelmuller and Mohr (1985) also reported that at least two photoreceptors are responsible for the anthocyanin synthesis in *Sorghum bicolor* and milo (*Sorghum vulgare* Pere.) seedlings.

Transient light activation of C1-sh-GUS was detected in all tissues tested including protoplasts (Figure 14), developing embryos (Figure 19), germinating embryos (Figure 40), roots and leaves (Figure 17). That C1-sh-GUS can be activated by light in leaf tissues offered a convienent and continuous supply of experimental material. Because the endogenous *C1* is normally only expressed in scutellum and aleurone layer of the seed in nature and not normally expressed in roots or leaves, this result indicates that not all elements of proper developmental control of light regulation are recovered in the transient expression assays. This is one of the major disadvantages of a transient expression assay (Katagiri and Chua 1992). On the other hand, we did find tissue specific activation of VP1 and negative regulation in our system.

## Localization of Light Responsive Sequences in *C1* Promoter

The first step in localizing the light response element was to define the minimal light responsive *C1* promoter. For this purpose, a series of 5' deletions of the promoter region of C1-sh-GUS were tested. As shown in Figure 24, mutants with deletions upto position -130 (D30) relative to the transcription start site remained light responsive while deletions upto -48 (D40, the minimal *C1* promoter with *sh1* intron) lost the response. This indicates that there exists an essential cis-element within this 83 bp region between -130 (D30) and -48 (D40). The -130 promoter construct (D30), however, has no VP1 and ABA response (Figure 23).

Because the G-box motif has been implicated in responses to several environmental stimuli including the UV light response complex defined in chalcone synthase promoter in parsley (Block et al. 1990), we tested the possibility that G-box-like sequences may be involved in light regulation of C1-sh-GUS.

The 609 bp promoter region of C1-sh-GUS contains three G-box-like sequences. The motifs include CATACGTTG at -176, GCCACGTAGTTA at -93 and CGCACGTCGA at

-44. A copy found at -93 (5'-GCCACGTAGTTA-3') is within the minimally responsive promoter defined by the terminal deletions (Figure 24).

To determine whether the G-box-like sequences are responsible for light regulation and to test the possibility of functional redundancy of elements in *C1* promoter, the corresponding internal deletion were made in each G-box-like sequence and the mutants tested in the bombardment assay. D54 deleted sequences from -179 to -171 (5'-GTTGCCCTT-3'), D53 deleted from -104 to -93 (5'-GCCACGTAGTTA-3' ) and M53, introduced a 2 bp site-directed mutation in the center of ACGT motif in the G-box-like sequence at -93.

As shown in Figure 25B, C, D, the G-box-like sequence at position -93 (5'-GCCACGTAGTTA-3' ) has a critical effect on light activation. Interestingly, the data show that a 2 bp change within the ACGT motif (M53, from ACGT to AACT) totally abolished the light response of *C1* full length promoter (609 bp). These results suggested that the critical G-box element at -93 is not functionally redundant with the other G-box-like elements in light regulation. This situation differs from what has been observed for other light regulated genes such as *rbcS-3A*, *chs* and *cab* genes (Block et al. 1990, Green et al. 1987, Ha and An 1988). For example, a replacement of two G residues in Box II of the -166 *rbcS-3A* promoter reporter construct abolished the light response (Kuhlemeier et al. 1988). However, the Box II and Box III elements are dispensable in a -410 promoter chimeric gene because there are homologous sequences (Box II* and Box III*) upstream from -166 (Green et al. 1987).

Similar light responses are observed in protoplasts, embyros and leaf tissues (Figure 25), indicating that the same cis-elements function in these tissues.

The third G-box-like sequence at -44 (-58 GGCCACGCACGTCGA -44 ) does not affect light regulation, however mutations in this element caused a significant increase in both basal and light-treated expression activity suggesting a possible negative regulatory role.

## Structure of the Light Response Element in *C1* Promoter

We then determined whether the G-box sequence at -93 alone is sufficient for light regulation of *C1*. To test this, multiple copies of the G-box element (GCCACGTAGT) were cloned in front of 35S minimal promoter (-46). In total 15 different constructs including monomer to pentamer configurations in both orientations were tested by electroporation and bombardment. None of the constructs showed consistent positive light activation (Figure 26). This indicates that for light activation the cis-element needs more sequences than just a G-box and flanking sequences. In other words, the G-box is necessary, but not sufficient for the light response.

In order to further delimit sequences in the *C1* promoter that are required for light regulation, a series of site-directed and internal deletion mutants were made in the D20 promoter (-235 relative to transcription start site) and tested for light activation. As shown in Figure 27, the 5' boundary of the light element is defined by the 2 bp change in the mutant 7677 which totally abolished light activation. Again a 2 bp change is sufficient to abolish light responsivity of the 235 bp long promoter.

This result indicates that the 5' end of the light cis-element extends 10 bp upstream of the G-box element. These results together with the effects of the internal deletion mutants Id6 and D20dNS localize the minimal light cis-element between -116 to -58 (Figure 27).

To determine whether single copy of the minimal light response region is able to confer light regulation, three test fragments that began at the same 5' end (-116) defined by site-directed mutant 7677 and extended to include progressively more 3' sequences were constructed. They are the 15 bp length TCLE (GGCCTCAACTCGGCC), 25 bp length SCLE (GGCCTCAACTCGGCCACGTAGT) and 72 bp length LCLE (from -130 to -59 of *C1* promoter) constructs. One copy of each was placed in front of 35S TATA (-46) and assayed in leaf and embryos bombardment experiments. Unexpectedly, none of the heterologous reporters were light-activated, even the 72 bp length LCLE failed to consistently confer light regulation to the 35S core promoter (Figure 28). Sequences downstream of -58 proved to be nonessential (D20DNS and D30DNS have positive light activation), whereas the same 72 bp in the original *C1* reporter context (D30DNS and D30) can be light activated (Figure 32). Therefore, it is not likely that the light cis-element requires more sequences for light activation.

These results suggest that there are other requirements in addition to the light cis-element for light response. We identified three possibilities: 1) The spacing between the light cis-element and TATA initiation complex may be critical; and that when we engineered the TCLE, SCLE, and LCLE constructs this crirical spacing was interrupted. 2) The original *C1* TATA sequence itself may have a specific role in this light regulation which can not be replaced by 35S TATA. For example the pea *rbcS-3A* TATA (from -50 to +15) is itself a light responsive element (Kuhlemeier et al. 1989). 3) The *sh1* first intron in 5' untranslated region of the original C1-sh-GUS may be necessary for light activation.

With respect to the first possibility, the distance between ACGT motif and the TATA box is 63 bp and 84 bp in C1-sh-GUS and LCLE, respectively. Kuhlemeier et al. (1987) have shown that insertion of as much as 66 bp between the *rbcs-3A* light cis-element and heterologous 35S TATA did not change light regulation and tissue specific expression pattern, neither did it change the transcription start site. Furthermore, the activities of Id4, Id5, D00DNS, D20DNS and D30DNS internal deletion constructs showed that the -58 to -45 sequence per se and altered spacing (by 15 bp shorter) between TATA box and the upstream elements do not influence light activation (Figure 22, 29, 37, 39). For this reason it is unlikely that altered spacing between the cis-element and TATA is the problem.

To address the possibility that essential sequences may be located in the TATA and core promoter region of *C1* we replaced the core TATA of D30 with the 35S TATA and transcription start site (Figure 29). The normal light activation of this construct (D3035S) rules out the possibility that there are special properties of *C1* TATA and core promoter region.

Finally one remaining feature that is common to all of the light activated minimal constructs is the presence of the *sh1* first intron in the 5' untranslated region of the GUS transcript. Therefore, we cannot exclude the possibility that the intron is necessary for the light response. Although the mechanism by which the *sh1* intron enhances transient gene expression remains poorly understood, it is unlikely that the intron has a specific role in light activation. The *sh1* gene itself and *sh2* with *sh1* first intron are not directly light regulated (Figure 30). A variety of unrelated promoter constructs which include the *sh1* intron in similar contexts showed no light activation in our system (Figure 15). The existence of specific *C1* promoter mutants which block light activation ( i. e. D53, M53, Id6) without affecting VP1 and/or ABA regulation, as well as the non-responsive D40 construct, clearly demonstrate that the intron is not sufficient for light activation in the context of the *C1* promoter.

We then put the 25 bp SCLE (GGCCTCAACTCGGCCACGTAGT) in front of the original minimal *C1* TATA (-48 ) with the *sh1* first intron, to see whether there is a smaller version of light cis-element. The construct fails to confer light activation (Figure 31). Therefore, the light cis-element in the *C1* promoter is 57 bp located between -116 and -59. This 57 bp cis-element functioning in both embryos and leaves, and also has the ability to respond to UV light (Figure 32).

As shown in Figure 23, this 57 bp region includes a dyad sequence (GGCCTCAACTCGGCC), which overlaps an adjacent G-box motif. This structure resembles the Box I and Box II (Unit I) element of the *chs* gene promoter of parsley (Block et al. 1990, Schulze-Lefert et al. 1989). Both are composed of an ACGT motif adjacent to a GGCC-------GGCC palindromic sequence spaced 7 bp apart and a symmetric TCAACT motif. Block et al. (1990) have shown that the 7 bp spacing is critical for the light expression of parsley *chs* gene. In the case of *C1* gene, additional sequences located 3' end to the structure are also necessary (Figure 31).

## Negative Regulation Element in *C1* Promoter

In defining the light response cis-element, we uncovered evidence of a negative regulatory element in the *C1* promoter. As mentioned above, there are 3 G-box-like sequences in *C1* promoter and their relevance to different stimuli was tested individually. The distal copy at -176 does not play a role in VP1, ABA or light regulation. The second G-box-like sequence at -93 (GGCCACGTAGT) is part of the light cis-element as shown above. The third element at -44 (CGCACGTCGA) just proximal to the TATA box (at -29) does not affect light or VP1/ABA regulation but may have a negative regulatory role in light activation. When this G-box-like motif is deleted, light activation still exists, but the expression levels in both dark and light-treated samples increase significantly (Figure 34). The dark expression of G-box deleted mutants (DS and DNS) is greater than the light-treated wild type (D00) level. This activity was used as a criterion to define the negative element. We then tested wild type and *vp1* mutant embryos to determine whether VP1 has any influence on this negative regulation. As shown in Figure 35, wild type and *vp1* mutant embryos have similar responses, indicating VP1 function is independent of this negative regulation.

## Cooperation of the Negative G-box Element with an Upstream Region

We then determined whether the proximal G-box and its flanking sequences were sufficient for inhibition of transactivation. To test this, we took out a large fragment from *C1* promoter (31 bp length 5'-GGCCACGCACGTCGACCGCGCGTGCATTT-3') containing the G-box and its flanking sequences and inserted it into the full length 35S promoter (pBI 221) at EcoRV site (-90) in both orientations to see whether it can suppress the constitutive expression of 35S promoter. As shown in Figure 36, down-regulated expression is not detected in dark or light-treated embryos. This could mean that the negative element has its own unique requirements for function.

However, tests of a set of mutants (D10dNS, D20dNS, D29dNS and D30dNS) revealed an additional level of complexity in that the negative cis-element at -44 has a negative effect only when a far upstream region is also present. As shown in Figure 37, the effect of the Nae I - Sal I deletion (13 bp) depended upon the length of *C1* promoter. When the *C1* promoter is less than 235 bp (D20DNS, D29DNS and D30DNS), the derepression effect of the NS sequence disappeared although the light activation was still present. This indicated firstly that the negative element is separable from the light cis-element and secondly, that in order for the cis-element at -44 to function as a negative regulator, it must cooperate with a upstream region which is at least 190 bp away. However, the reciprocal construct with the upstream region deleted but retaining the negative G-box element at -44 (D10 and D20) does not show derepressed expression (Figure 24).

## Tissue Specific Expression of VP1/ABA and Negative Regulation

From the above results it is clear that within 150 bp upstream of the transcription site of *C1* gene, there exist at least four different cis-elements: VP1, ABA, light and negative regulatory elements. As mentioned earlier, *C1* gene is under strict tissue-specifc regulation and is expressed only in seed tissues (aleurone layer and scutellum tissue) in vivo. One potential disadvantage of using transient expression assay (like electroporation and particle bombardment) is the possibility that some mechanisms of tissue-specific regulation may not be recovered. This is due to several limitations in the transient expression system. (1) Multiple copies of the reporter construct (C1-sh-GUS) are introduced into a single cell which may titrate out repressors or interact with minor activators that have a low binding activity. (2) The reporter DNA is not integrated into the genome. Therefore, the chromatin structure of the reporter gene may differ from that of the endogenous nuclear gene. (3) Multiple copies of the effector constructs (35S-sh-Vp1) may generate a concentration of VP1 higher than the normal physiological concentration. (4) Translational control can not be addressed for either the effector or the reporter genes. On the other hand, a tissue specific (embryo) expression pattern was observed for VP1 activation and the negative regulatory element.

### VP1 Protein Activates C1-sh-GUS in Embryos but not in Leaves

As shown in Figure 4, C1-sh-GUS can be activated by light in different tissues including those where *C1* is not normally expressed in vivo, i.e. the leaf. However, while the VP1 protein can activate the C1-sh-GUS in developing embryos, cobombardment of 35S-sh-Vp1 and C1-sh-GUS produced no activation in the leaf (Figure 38). In contrast, Em-GUS is activated by VP1 in both embryo and leaf tissues (Cocciolone and McCarty unpublished data). Therefore, in addition to VP1 protein, additional seed specific factors may be required for the *C1* gene to be activated by VP1 protein.

## Seed Specific Negative Regulation

The inhibitory effect of the -44 region was also evident only in seed tissues. When the same mutants (DNS and DS) were tested in leaves, basal activities were similar to the intact promoter and normal light activation was observed (Figure 39). This result indicates a tissue specific function of the negative regulation which in turn suggests that additional seed specific factors (repressor) may be necessary for negative regulation in the seed tissues.

It is possible that this seed specific negative regulation of *C1* may be linked to the phenomena of induction or derepression of the anthocyanin pathway in the aleurone layer during the germination phase (Chen and Coe 1977), if we assume that the putative repressor is only present in developing seeds. However, the results show that negative regulation persists in the dark and light germinating embryos which suggests that the putative repressor is also present in the germination phase (Figure 40).

## Sequence Comparison between *C1* and *pl* Promoter

The *C1* and *Pl* genes have been classified into one gene family based on the fact that they encode functionally equivalent proteins which share homology to the DNA binding domain of *myb* oncoproteins (Paz-Ares et al. 1987). Genetic studies in maize indicate that *C1* regulates the anthocyanin structural genes in the aleurone layer of the kernel and the *Pl* gene regulates the same pathway in plant (vegetative and floral tissues) but not in the seed.

The *pl* alleles can be classified into two groups: Dominant (*Pl*) alleles lead to light-independent anthocyanin synthesis, and recessive *pl* alleles lead to light-dependent ("sun-red") pigmentation in which only the portions of the plant body exposed to light become pigmented (Cone et al. 1993b). Based on the fact that both *C1* and *pl* require light for anthocyanin synthesis, it has been proposed that *C1* and *pl* are functionally similar genes that arose from duplication of a common light-regulated ancestor. This proposal is further supported by evidence that the genes encode proteins with more than 90% amino acid identity (Cone et al. 1993).

The 5' flanking promoter regions of *C1* and *pl* are also highly conserved. When compared the cis-elements defined in *C1*, the G-box and dyad structure required for the light cis-element and the G-box for negative regulation are well conserved in the *pl*. However, 5 nucleotides out of 17 differ within the cis-element that is critical for VP1/ABA activation (Figure 41). For this reason the expectation is that *pl* gene should be light activated, however due to the 5 bp changes in the ABA/VP1 cis-element it may not be responsive to ABA or VP1 in seed tissues.

To address this question we switched the upstream regions between *C1* (D00) and *pl* at the Sal I site. As shown in Figure 42, *C1* and *pl* can be light activated equally well; however, while *C1* can be activated by VP1 very well in the seeds, the *pl* has no response to VP1.

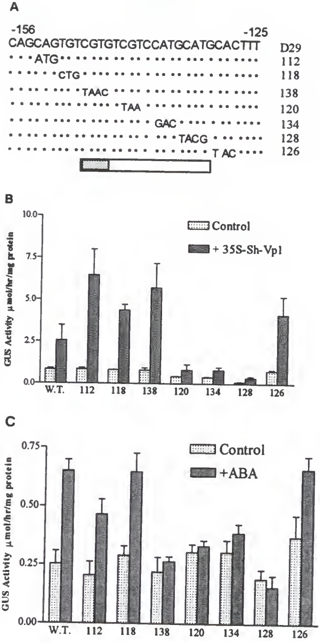

Figure 11. Localization of *C1* promoter sequences critical for ABA regulation and VP1 trans- activation in the protoplast electroporation assay. (A). A series of site-directed mutants were made between -156 and -125 in the promoter of C1-sh-GUS and the responses of these mutants to VP1 (B) and ABA (C) deter -mined. The blank rectangle on the bottom of (A) represents the sequences critical for both ABA and VP1; and the dark rectangle represent the sequence specifically critical for the ABA regulation.

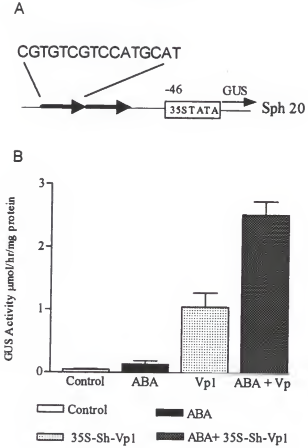

Figure 12. The 16 bp sequence is sufficient for both VP1 and ABA regulation. Top, the dimer of the sequence important for ABA/VP1 activation in the minimal 35S (-46) promoter reporter construct. Bottom, the responses of the reporter construct to ABA and VP1 in the protoplast electroporation.

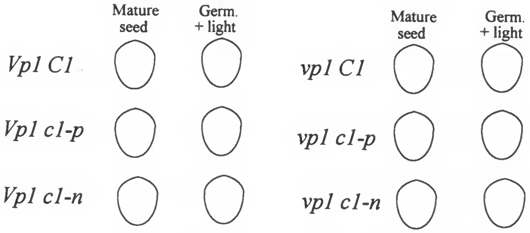

Figure 13. Light exposure can induce the synthesis of anthocyanin even when the upstream activator gene *Vp1* is in recessive status. The mature seed in *vp1* mutant background should be viviparous (adapted from Hattori et al., 1992).

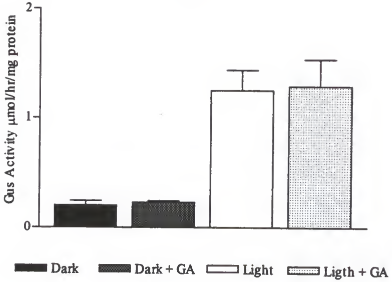

Figure 14. Light activation of C1-sh-GUS following electroporation ofdark-grown suspension cell protoplasts. Gibberllic acid (10 µM) has no effect on C1-sh-GUS or its light regulation.

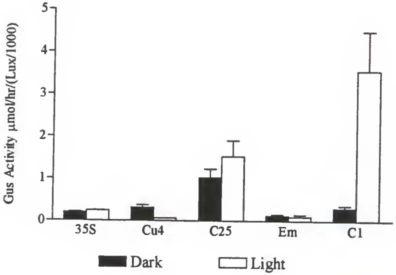

Figure 15. The specificity of C1-sh-GUS light response relative to four different promoter reporter constructs with or without the *shrunken* first intron in leaf bombardment. 35S, 35S promoter-GUS; Cu4, *Adh1*promoter -*adh1*intron 1-GUS; C25, Ubiquitin promoter -*ubi* intron 1-GUS; Em, *Em* promoter-GUS.

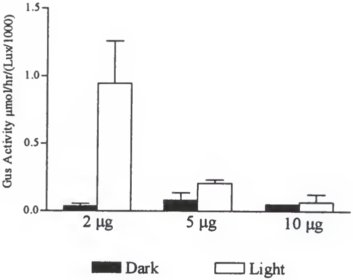

Figure 16. Effect of reporter DNA (C1-sh-GUS) concentration on light activation in particle bombardment assays of leaf tissues from light-adapted seedlings.

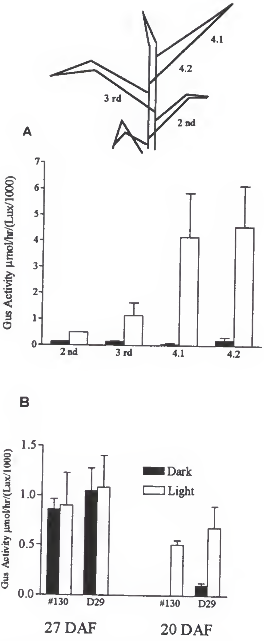

Figure 17. Physiological ages of the material affect sensitivity to light. (A) Light responses of C1-sh-GUS (D00) in different leaves from the same seedling. (B) Light responses of D29 and mutant # 130 in *vp1* mutant embryos with 20 and 27 DAP. D29 has only -157 bp in *C1* promoter region. #130 is a site-directed mutant of D29 with the changes of GCGC to TTAA at -40.

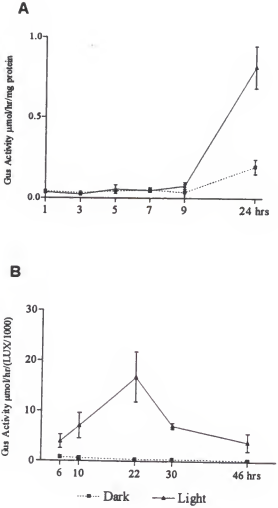

Figure 18. Time course responses of the light regulation of C1-sh-GUS (D00) in (A) electroporated protoplasts and (B) leaf bombardment, respectively.

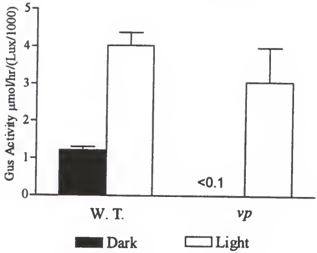

Figure 19. Light activation of C1-sh-GUS is independent of VP1 regulation. Both wild type and *vp1* mutant developing embryos from the same ear give positive light responses after bombardment with the D00 construct.

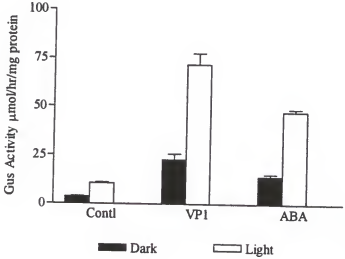

Figure 20. The synergestic interaction between light, VP1 and ABA activation of C1-sh-GUS (D00) in electroporated protoplasts.

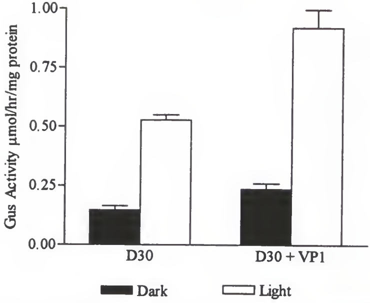

Figure 21. D30 (-130) reporter construct without the cis-element for VP1 and ABA (located at -147 to -132) is still light responsive in electroporated proto -plasts.

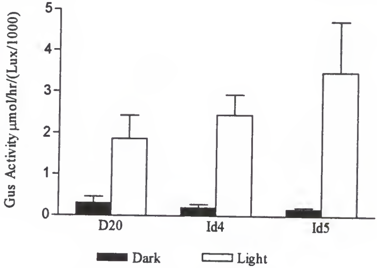

Figure 22. The cis-element involved in VP1and ABA regulation is not a part of the light responsive element. D20 has 235 bp in the promoter region of C1. Id4, the internal deletion mutant with sequences -151 to -147 deleted which is critical for ABA regulation and Id5, with deletion from -136 to -131 which is important for VP1 activation. Both mutants do not affect the light activation in leaf tissue.

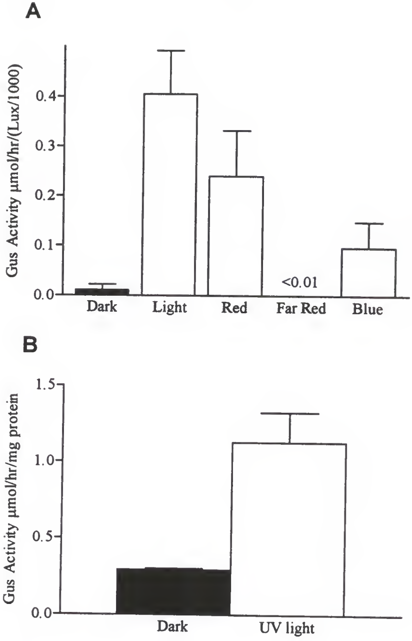

Figure 23. The effects of different light wavelengths on C1-sh-GUS implicate involvement of multiple photoreceptors including phytochrome. (A) Bombard -ment of C1-sh-GUS (D00) in leaf and illuminated with different wavelength. Light, white light from a slide projector; Red, red light (> 600 nm); Blue, blue light (400 ~500 nm); Far red, far red light (> 700 nm). (B) The UV light effect on C1-sh-GUS (D00) in electroporated protoplasts.

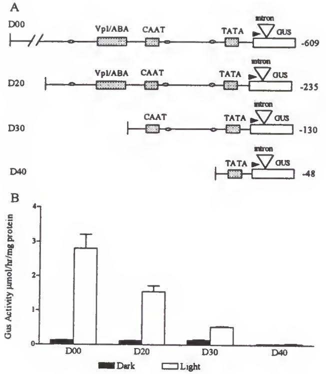

Figure 24. Localization of *C1* promoter sequences required for light regulation. (A ) A series of 5' deletions of C1-sh-GUS were tested for light regulation. (B) The responses of the 5' deletion mutants to light in leaf bombardment. D30, a deletion upto -130 relative to the transcription initiation site is not ABA/VP1 responsive (see Fig. 21), but retains light responsive.

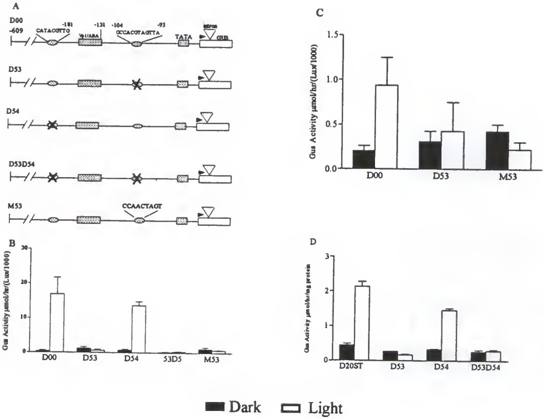

Figure 25. The responses of the G-box internal deletion (D53, D54, D5354) and multiple base changes mutant M53 specify the involvement of the G-box at -93. (A) Constructs show the positions of each individual G-box, the internal deletion mutants and the 2 bp changed mutant. The light responses of these mutants are shown in (B) leaf bombardment, (C) developing embryo bombardment and (D) electroporated protoplasts.

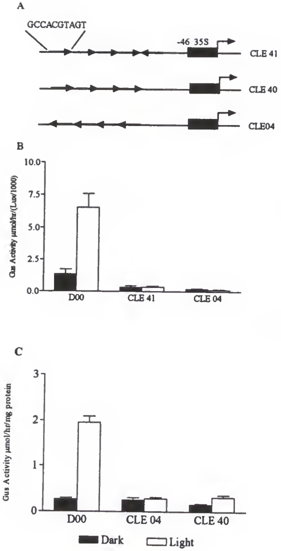

Figure 26. G-box-like sequence is necessary but not sufficient for light activation. (A) constructs that contain only G box (GCCACGTAGT) multimer in front of the 35S minimal promoter (-46) and (B) their light responses in leaf bombardment and (C) electroporated protoplasts, respectively.

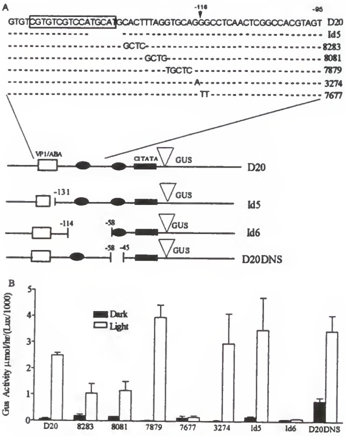

Figure 27. Resolution of the light responsive domain in *C1* promoter. (A) A series of site-directed mutants 5' to the G-box (GGCCACGTAGTTA) at -93 and internal deletion mutants (Id5 and Id6) were tested for the light regulation in leaf particle bombardment. (B) The light response of these mutants defined 5' boundary for the light element (7677) which is 10 bp more 5' to the G-box (CGGCCACGTAGT) at -93, and the light response of D20DNS defined the 3' end of the element.

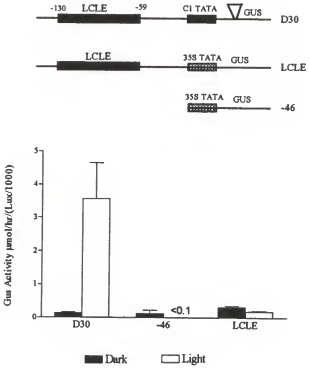

Figure 28. The largest potential light cis-element, 72 bp length LCLE does not confer gain of function to the minimal 35S (-46) promoter.

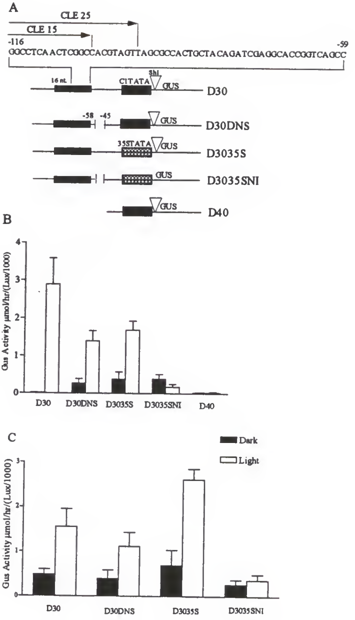

Figure 29. Gain of function experiments using the light response element. (A) The constructs combine a 74 bp minimal response element with the *C1* TATA or *35S* TATA core promoters with or without the sh1 first intron. (B) and (C) show the light responses of these constructs in the leaf and developing embryo tissue, respectively. The CLE15 and CLE25 indicated in (A) were the oligonucleotides tested in minimal *C1* promoter (D40) (See Fig. 31).

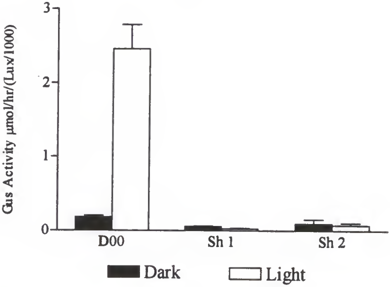

Figure 30. The *Sh1* and *Sh2* promoter with *sh1* first intron can not respond to light in leaf bombardment assays. Sh1, *sh1* promoter-*sh1* intron 1-GUS; Sh2, *sh2* promoter-*sh1* intron 1-GUS (Sh1 and Sh2 plasmid kindly provided by Dr. L. C. Hannah, Univ. of Florida)

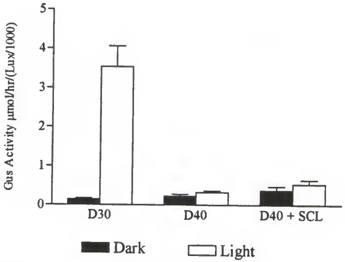

Figure 31. The 25 bp length cis-element (GGCCTCAACTCGGCCACGTAGT) which contains the dyad structure and G-box motif in front of original *C1* TATA and the *sh1* intron (See Fig. 29 ) can not be light activated.

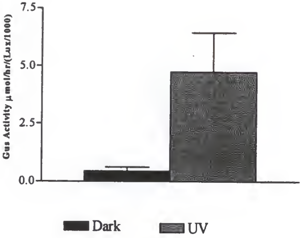

Figure 32. The minimal light resposive construct (D30DNS) still respond to UV light in leaf bombardment.

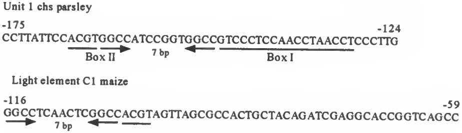
Unit 1 chs parsley
-175
-124
CCTTATTCCACGTGGCCATCCGGTGGCCGTCCCTCCAACCTAACCTCCCTTG
Box II
7 bp
Box I
Light element C1 maize
-116
-59
GGCCTCAACTCGGCCACGTAGTTAGCGCCACTGCTACAGATCGAGGCACCGGTCAGCC
7 bp

Figure 33. Sequence comparison between the light cis-element of *C1* gene in maize and the UV boxes of the *chs* gene in parsley

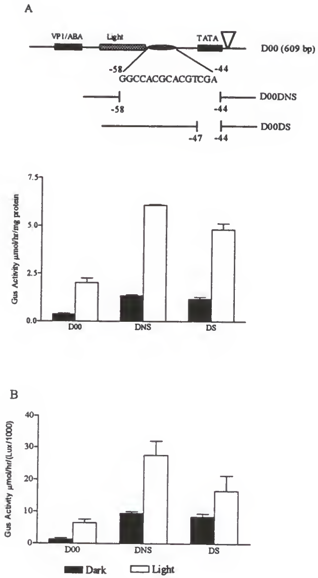

Figure 34. The G-box-like sequence at -44 has a negative effect on light activation. (A) Top, the constructs of the G-box deletion mutants, DNS and DS. Bottom, the light responses of the G-box mutants in protoplasts from embryo-derived cell suspension, and (B) in developing wild type embryos.

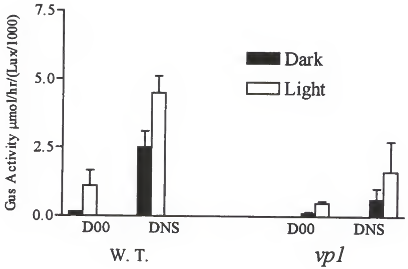

Figure 35. The negative regulation function of DNS is detected in both wild type and *vp1* mutant developing embryos.

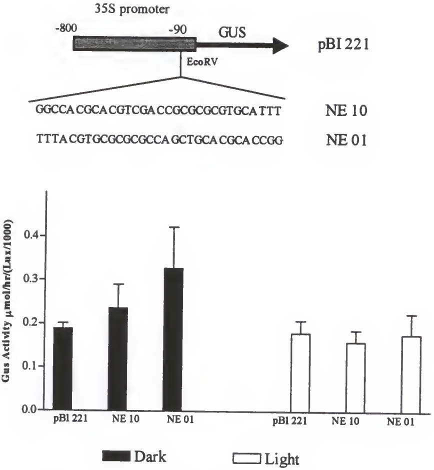

Figure 36. The putative negative cis-element has no affect on *35S* promoter function in dark and light-treated developing embryos. Top, a 31 bp sequence including the negative G-box placed in full length 35S promoter in both orientations. Bottom, both NE 10 and NE 01 have the same expression level as the pBI plasmid.

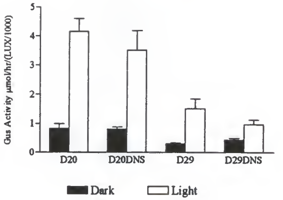

Figure 37. An upstream region is necessary for the negative regulation of *C1* promoter in embryos. D20DNS (235 bp) and D29DNS (157 bp) with the same G-box internal deletion as D00DNS (609 bp) have no negative regulation ability. Compare the same 13 bp deletion on full length C1 promoter (DNS and DS) in Fig. 24.

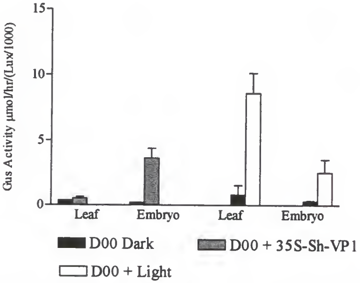

Figure 38. Tissue specificity of transactivation by VP1 protein in comparison to light activation of C1-sh-GUS. Embryo, developing embryos of *vp1* mutant. Leaf, the latest fully expanded leaf of wild type (W22) seedling.

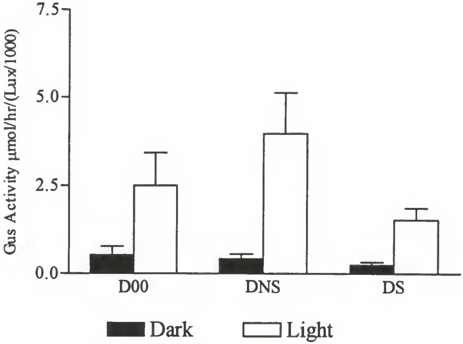

Figure 39. The negative regulation of DNS and DS mutants is not detected in the leaf tissue, but maintains a similar level of light activation as wild type C1-sh-GUS.

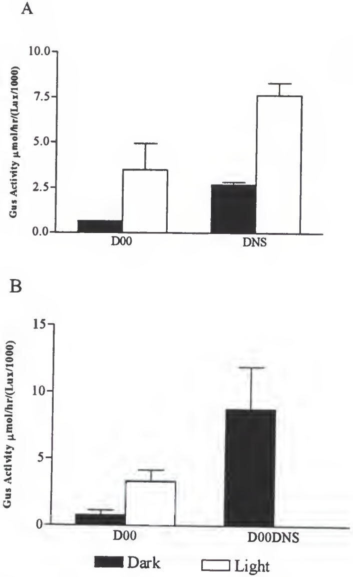

Figure 40. The negative regulation of DNS can function in both (A) dark- and (B) light-treated germinating embryos. The wild type kernels were imbibed in water for 48 hrs under dark or light before bombardment.

```
                              VP1/ABA
C1        CAGCAGTGTCGTG-T-CGTCCATGCATGCACTTTAGGTGCAGTGCAGGGCCT
          CAGCA TGTCGTG   CGT C TGC TGCA TTTAG      TGCA GGCCT
pl-McC    CAGCACTGTCGTGGTCCGTACGTGCCTGCAGTTTAGCGTT--TGCATGGCCT
            Light G-box
C1        CAACTCGGCCACGTAGTTAGCGCCACTGCTACAGATCGAGGC------ACCG
          CAACTCGGCCACGTA    GCG CA T CTAC     GAG        ACCG
pl-McC    CAACTCGGCCACGTA----GCGGCAGTTCTACG----GAGTGAAGCTAACCG

                            Negative G-box          TATA box
C1        GTCAGCC---------GGCCACGCACGTCGACCGCGCGTGCATTTAAATACGCC
            CAG           GGCCACGCACGTCGAC GCG   TGCATTTAAATACGCC
pl-McC    ACCAGTTGGGGGCCTGGGCCACGCACGTCGACAGCG----TGCATTTAAATACGCC
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Figure 41. Sequence comparison of the cis-elements contain in *C1* and *pl* promoters. In the critical sequences for VP1/ABA there are 5 bp differences between *C1* and *pl*, which make *pl* unresponsive to VP1 and ABA activation. Although the light G-box is completely conserved in *C1* and *pl*, the G-box alone is not sufficient for light activation (see Fig. 26).

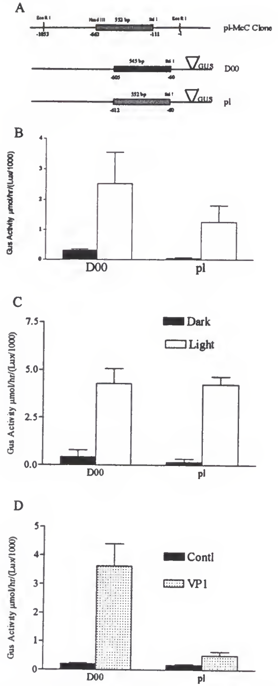

Figure 42. The universal light activation of *C1* (D00) and *pl*; and the contrast in VP1 activation between D00 and pl. (A) constructs of D00 and pl. The pl is derived from switching the comparable region in *pl-McC* (kindly provided by Dr. K. Cone, Univ. of Missouri) to D00 at Sal I site. (B) and (C) the light responses of D00 and pl in embryos and leaf tissues, respectively. (D) co-bombardment of 35S-sh-Vp1 can activate the D00 but not the pl construct in embryos.

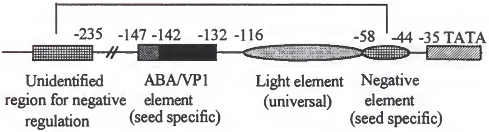
-235
-147
-142
-132
-116
-58
-44
-35
TATA
Unidentified region for negative regulation
ABA/VP1 element (seed specific)
Light element (universal)
Negative element (seed specific)

Figure 43. Summary of the cis-elements defined in*C1* promoter. The ABA/VP1 element is composed by Sph element and an extra CGTGT motif. The light cis-element has G-box, dyad and symmetric structural feature. The negative element also contains a G-box-like sequence and an upstream unidentified region that is necessary for the function.

# DISCUSSION

By studing the transient expression of introduced DNA sequences into protoplasts by electroporation and into intact tissues by particle bombardment, we are able to show that the region -147 bp upstream of transcription start site in *C1* promoter includes at least four different cis-acting elements. They are ABA (-147 CGTGTCGTCCATGCAT -132), VP1 (-142 CGTCCATGCAT -132), light (from -116 to -59) and a negative regulatory element at -44 (Figure 43). The cis-acting element for ABA/VP1 is resolved into the Sph element (CGTCCATGCAT) which is required for the ABA and VP1 responses and an upstream element (CGTGTC) which is specifically required for ABA regulation in the absence of VP1 over-expression. This is consistent with our previous finding that the GTGTCGTGTC repeat which distinguishes wild type *C1* alleles from the *c1-p* alleles is necessary for ABA regulation (Hattori et al. 1992). The present results indicate that the C residue (underlined) contributed by the upstream copy is the critical difference between these alleles. This Sph element (TCCATGCAT) overlaps an extensive RY motif (CATGCATGCAC) that has been implicated in seed specific expression of genes (Dickinson et al. 1988) and bears resemblance to one component of the GA regulated low pI α -amylase promoter (TCCATGC) (Gubler and Jacobsen 1992, Lanahan etal. 1992).

Interestingly, both the *C1* and α- amylase genes have aleurone specific expression in endosperm.

We present evidence that light (including UV, Blue and red light) can drive the expression of a *C1* promoter chimeric gene in leaf tissues under normal physiological conditions, i. e., light-dark diurnal cycles. The activation of *C1* promoter apparently responds to multiple photoreceptors including phytochrome (Figure 23). Phytochrome regulation is common to light activation of *chs* and *rbcS* genes. The fact that phytochrome is more abundant in relatively young and rapidly expanding tissues may support the results in Figure 17 which show that more apparant light activation is observed in younger leaf tissue than in older ones. Based on the fact that far red light can not induce expression, we can classify the light response of C1-sh-GUS, as in the majority of known light response, into the low fluence (LF) response category (Kaufman et al. 1984).

The light responsive cis-acting elements of *C1* gene are localized to a 57 bp fragment. This 57 bp includes a dyad sequence (GGCCTCAACTCGGCC), and an adjacent ACGT motif. The palindromic sequence (GGCC-------GGCC) has a symmetric sequence (TCAACTC) embedded within it. These features are reminiscent of the UV light cis-element (Unit I) of the *chs* gene promoter of parsley. Block et al. (1990) have shown that the 7 bp spacing of the GGCC motif is critical for the light expression of parsley *chs* gene. In *C1* gene, additional sequences located 3' end to this complex structure are also necessary (Figure 17). In the case of the *chs* gene the shortest cis-element to have been tested in a gain of function assay is 226 bp long.

It is interesting to note that one of the maize chalcone synthase genes (*C2*), which is regulated by the *C1* gene in the aleurone and scutellum, also includes a Box I like element in the promoter region (Franken et al. 1991). It has been shown that the maize *C2* mRNA can be light activated in seedlings with all possible combinations of the upstream regulatory genes (*-r/g*, *B/b* and *Pl/pl*) (Taylor and Briggs 1990). This suggests that a common light regulatory pathway may activate both the regulatory and structural genes in the pathway and that unlike developmental control, light regulation of the pathway may not be hierarchial.

Another notable feature of the light cis-element in *C1* is that it does not have mutiple functionally redundant elements, which have been observed in other light activated genes such as the Box II, Box III, Box II* and Box III* motifs of the *rbcS-3A* promoter (Green et al. 1987) and the Unit I ( Box I and Box II) and Unit II (Box III and Box IV) elements of the parsley *chs* gene (Block et al. 1990). The redundancy of light-responsive elements are proposed to ensure the maximum light activated expression under various lighting environments through the cooperative binding of the cognate activators to multiple light elements. In *C1* gene of maize our data show that a two bp change in the critical region of light renders the full length (609 bp) promoter light insensitive indicating that there are no redundant light regulatory elements (Figures 25, 27).

A key feature which distinguishes the VP1 and light responses that we detected in the transient expression assay system is the contrast in tissue specificity. VP1 transactivation of C1-sh-GUS is detected only in suspension culture protoplasts and seed tissues, whereas, this same construct is light activated in all tissues tested including leaf and root tissues. The endogenous *C1* gene is expressed exclusively in embryo and aleurone tissue and does not functionally overlap with the plant specific *pl* gene in light regulation of anthocyanins in nonseed tissues (Cone et al. 1993). Therefore, although both light and VP1 regulation of the *C1* gene are normally seed specific responses, our results indicate that the tissue specificity observed in each case is determined by different mechanisms.

The universal light activation mediated by the minimal *C1* light response element is consistent with the fact that these sequences are highly conserved in the *pl* promoter; whereas, the VP1/ABA response region is divergent in *pl*. Our results show that the 5 nucleotide difference in the region corresponding to the VP1/ABA response element of *C1* in the *pl* promoter are sufficient to make *pl* unresponsive to VP1 activation in seed tissues (Figure 42). This together with the indication that additional seed specific factors are necessary for VP1 activation (Figure 38) sheds some light on the differential tissue expression of *C1* (in scutellum and aleurone layer) and *pl* (in vegetative and floral tissue of the plant). However, the light activation of *C1* and *pl* that occurs in seed and plant tissues points out some missing pieces in our understanding of tissue specific regulation. The determinants of tissue specificity for light regulation may reside in 5' or 3' flanking regions that are not included in our reporter construct or even within the transcribed region as in the case of the pea ferredoxin gene (Gallo-Meagher et al. 1992). On the other hand, modes of control that involve chromatin structure or other epigenetic mechanisms are not likely to be recovered in a transient expression assay.

The G-box-like sequence (CGCACGTCGA) at -44 has a negative regulatory function that affects not only basal (dark) transcription but also light activated levels (Figure 35). In addition to the inhibition of transcription, this negative G-box sequence has three unique features. Firstly, its close proximity (at -44) to the TATA box (at -29) is such that components (TFII D, A, B, F, etc.) of the initiation complex might actually cover this sequence. Secondly, the inhibitory function of this G-box is dependent on an unidentified upstream region which is at least 190 bp away (Figure 34, 37). Thirdly, this negative function is seed specific (developing and germinating embryo) (Figure 34, 39, 40).

Although negative regulation of gene expression has been recognized to be at least as important as positive activation, plant negative response elements have so far only been described in pea *cab*, *rbcS*, oat *phyA3* and tobacco *cab-E* genes. In tobacco, the negative element was defined in a large far upstream region (from -1182 to -747) (Castresana et al. 1988). In the pea *cab* gene a 247 bp fragment (from -347 to -100) can suppress *nos* promoter expression in the root, whereas this same fragment acts as a positive light element and enhancer in leaves (Simpson et al. 1986). In the pea *rbcS-3A* gene three copies of Box II (GTGTGCTTAATATG) or one copy of Box III (ATCATTTTCACT) when placed behind the 35S enhancer (from -943 to -31) turn off the effect potentiated by the enhancer in the dark but not in the light (Kuhlemeier et al. 1987, Green et al. 1988). Interestingly, the Box II and Box III are the light responsive elements in the pea *rbcS-3A* gene. This means that the negative and positive light responsive elements overlap to within 12 to 14 bp. In the oat *phyA3* promoter the sequences from -80 to -70 (CATGGGCGCGG) are delineated as the repressor element (RE 1) (Bruce et al. 1991).

The RE 1 is immediately downstream of a positive light element (PE3, from -111 to -81) and very close to TATA (-35).

The negative element in *C1* promoter is structurally separate from but functionally interacts with the positive light, VP1, ABA responsive sequences. The constructs that derepress basal expression (DNS, DS and other DNS series mutants) have normal light, VP1 and ABA activation characteristics (Figure 34, 37, 39).

We addressed the possibility that the seed specific negative regulation element at -44 has a role in regulation of pigmentation during seed germination. One possibility is that a seed specific repressor or mechanism may be active only during seed development and maturation phase and that it inhibits the light effect on *C1*. This inhibitory effect may then be relieved by inactivation of the repressor during seed germination. In that case, the pigmentation of aleurone layer in the *vp1C1* and *c1-p* alleles would result from derepression of basal transcription plus light activation of *C1*. This would require that the derepression effect is only apparent in developing but not germinating seeds. This scenario is not supported by the results of Figure 40 which shows that dark or light germinating seeds still have negative regulation.

The combinational interaction between VP1 and ABA shows an additive effect in the original *C1* promoter where only one copy of the VP1/ABA cis-element is present (Hattori et al. 1992). However, a dimer of the VP1/ABA cis-element (Sph 20) shows a synergistic interaction of VP1 and ABA activation (Figure 12). Synergistic effects were also observed in light plus VP1 and light plus ABA treatments (Figure 20). This suggests that light treatment enhances VP1 and ABA activation of *C1* . This is consistent with the observation that these signals function synergestically in normal seed development. For example, wild type seeds grown in total darkness fail to accumulate anthocyanins even though VP1, *C1* and ABA are all present. Therefore, a light signal seems to be required in addition to VP1 and ABA signals for induction of the pathway during seed maturation. Conversely, the phenotypes of the *c1-p* allele and *vp1* mutants indicate that light alone is insufficient to activate *C1* in the absence of a functional ABA response element and the VP1 factor, respectively. These interactions suggest that the combinatorial arrangement of ABA, VP1 and light response elements in the *C1* promoter has a role in the intergration of these signals.

In summary, in addition to the transcriptional initiation complex on the TATA (-29), the proximal 120 bp of the C1 promoter contains four different functional cis-acting elements; they are, from the 5' to 3' direction, ABA, VP1, light and a negative regulatory domain (Figure 43). Thus, like *rbcS-3A* (Kuhlemeier et al. 1988), *phyA3* (Bruce et al. 1991) and 35S promoters (Philip and Chua 1990), the *C1* promoter has a modular regulatory structure. How these functional elements interact with each other during the different developmental phases of seed remains unknown. These may lie an understanding of the *c1-p* phenotype.

# SUMMARY AND CONCLUSIONS

Using protoplast electroporation and particle bombardment assays, we have identified the cis-acting elements for the regulation of *C1* by ABA, VP1 and light. The sequence from -142 to -132 (CGTCCATGCAT) is necessary and sufficient for VP1 activation, whereas a larger overlapping element from -147 to -132 (CGTGTCGTCCATGCAT) is necessary for ABA activation. A separate light (blue, red and UV) responsive cis-element located between -116 to -59, includes features similar to the UV response element identified in the parsley *chs* promoter. G-box-like sequence at -44 functions as a tissue specific negative element which can derepress the expression of basal, ABA, VP1 and light activated transcription in seed tissues. An unresolved upstream region is also necessary for this negative regulation. In contrast to the universal light activation, the VP1 activation of *C1* promoter is seed specific which, like the seed specfic negative regulation, suggests that additional tissue specific factors are required.

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